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14673

The Time Factor in the Production of Gastric Lesions on Low Calcium Diets.

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Gastric lesions develop in rats kept for 4 weeks on a low calcium diet. They occur exclusively in the antrum, the non-acid, mucus-secreting region of the glandular portion of the rat's stomach. The restoration to normal dietary calcium levels prevents the lesions.[1] The microscopic appearance of various gastric lesions together with a discussion of the normal histology of the rat's stomach has been given by Berg.[2] The fundus and rumen show defects, each characteristic for its location, on dietary deficiencies which have not as yet been definitely identified.[3,4]

Most of our experiments were based on a set 28-day period. There have, however, been indications that time is a distinct factor in determining the end results, and we have now extended the duration of experiments. The diet was essentially the same as previously described[1] (0.017% calcium and 0.41% phosphorus). Two groups of 10 and 12 animals respectively, matched as to weight, sex, and litter origin, were started simultaneously. The members of the first group were killed after 4 weeks; the others were left on the diet as long as 8 weeks.

Fig. 1 shows representative photographs of the stretched stomachs preserved in formalin.

As between the two groups, the mean number of lesions per rat did not differ essentially (22 vs. 24). The defects after 4 weeks appeared as well defined single lesions 1-3 mm in diameter. On the longer experiment, they appeared larger (2-5 mm) and many were confluent and irregular in shape. The central craters, also, were larger and more irregular and the degree of hyperplasia appeared greater. At the end of the 8-weeks experiment, many more craters were filled with blood clots than at the end of 4 weeks. In the former, the gastric contents always contained visible blood, whereas in the latter, gross evidence of bleeding was less frequent. In both groups, the lesions remained superficial, *i.e.*, did not penetrate beyond the submucosa. One of the objects of the longer experiment was to ascertain whether or not with

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[1] Zucker, T. F., and Berg, B. N., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 34.

[2] Berg, B. N., *Am. J. Path.*, 1942, **18**, 49.

[3] Sharpless, G. R., *Federation Proc.*, 1942, **1**, 192.

[4] Schiödt, E., *Acta med. scand.*, 1934, **84**, 456.

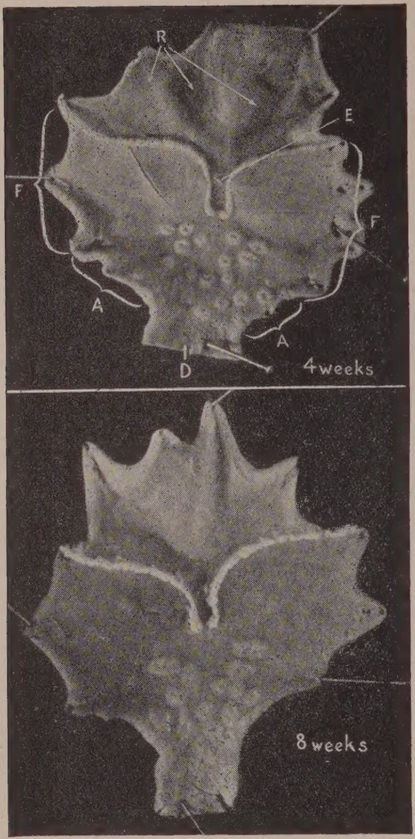

Fig. 1.

Rats' stomachs opened along the greater curvature, stretched out and preserved in formalin. Above the dividing ridge is the rumen (R) and below is the glandular portion consisting of fundus (F) and antrum (A). The lesions are restricted to the antral area. The division between antrum and fundus, while not so obvious in the reproduction, is easily distinguished in the specimen by the greater transparency of the former. Below the pylorus a small segment of duodenum (D) is shown. The esophagus (E) enters at the dividing ridge near the juncture of the three types of epithelial coats.

extended time the lesions would perceptibly approach the penetrating type.

It has been stated that 2 different deficient diets—deficiencies of calcium and B-complex respectively—will in 4 weeks' time lead to epithelial lesions, each characteristic of a definite region of the glandular portion of the stomach. Separate etiologies are indicated. It might of course also be the case that, with any common cause, the rate at which the process goes on may differ for the two types of epithelium found in the antrum and fundus respectively. Since the animals of the longer experiment were in large part exposed to the diet until practically moribund, and no fundus changes were produced, it seems unlikely that calcium deficiency *per se* will ever produce them.

Discussion. In the dog, Martin[5] has shown that prolonged marked calcium deficiency may produce hemorrhage in any part of the body, particularly the gastro-intestinal tract. Eppright and Smith[6] mentioned nasal hemorrhage incidental to calcium deficiency in rats. Boelter and Greenberg,[7] observing rats on low calcium diets, attributed paralysis of the hind legs to hemorrhage into the spinal canal and also record muscle hemorrhage. Light and Frey,[8] on the basis of rabbit experiments, believed that such paralysis is primarily due to fracture of vertebræ. We have seen loss of hind leg function (paralysis?) and muscle hemorrhage occasionally in the shorter experiments and found these symptoms quite pronounced as the 8-week experimental period neared its end. About half the animals were either moribund or dead before the full 56 days had elapsed.

Muscle hemorrhage in the lower extremities occurred with or without fractures in that region. This confirms the existence of a hemorrhagic tendency independent of fracture trauma. The circumscribed hemorrhagic areas in the gastric antrum may be secondary to necrosis. However, the existence of a more generalized hemorrhagic tendency should not be overlooked, nor should primary vascular changes be discounted entirely as initial causative local factors. We agree with Quick[9] that lowered blood calcium under such conditions (about 6 mg per 100 cc) cannot be looked upon as leading to a lessened coagulability which in analogy with Vitamin K phenomena could be held to be a factor in the hemorrhage described.

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[5] Martin, G. J., *Growth*, 1937, **1**, 175.

[6] Eppright, E. S., and Smith, A. H., *J. Nutrition*, 1937, **14**, 21.

[7] Boelter, M. D. D., and Greenberg, D. M., *J. Nutrition*, 1941, **21**, 61.

[8] Light, R. F., and Frey, C. N., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 256.

[9] Quick, A. J., *The Hemorrhagic Diseases . . .*, Thomas, 1942, p. 27 ff.

Summary. Keeping rats for longer periods of time on low calcium diets accentuates the ulcerative as well as the hyperplastic and hemorrhagic character of the antrum lesions as against the conditions produced in 4 weeks. It also brings out a generalized tendency to bleeding. No perceptible change, however, is produced with regard to penetration, the lesions remaining superficial.

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Tumor-bearing Chicks Hatched from Eggs Inoculated with Mouse Carcinoma.*

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Taylor, McAfee, and Taylor[1] state that chicks may hatch from eggs inoculated into the yolk with mouse mammary carcinoma.

Using a slightly modified Taylor technic,[2,3] fertile eggs were inoculated on the 5th day of incubation with minced, or pressed mammary carcinoma of strain A mice. All materials used came from tumors arising originally in this strain and having a variable number of transfers in the strain. Mouse transfers were done using sterile technic.

Tumor-bearing mice were killed with ether and the skin sterilized with iodine. Separate instruments were used for skin dissection and manipulation of the tumor. Pressed tumors were forced through muslin supported by a drilled, brass plate. Minced tumors were cut with very sharp fine scissors without addition of fluid. In both cases Tyrode solution was then added to give a suspension of about the same density as that used by Taylor.

The shell over the air sac was wetted with 70% alcohol and after drying was cut through with a round dental burr, turned by a high speed electric hand tool, care being used not to cut through the shell membrane. The membrane was wetted with alcohol and allowed to dry. The membrane was then burned through with a hot, tapering probe large enough to fit tightly into the hole in the shell, thus sterilizing the shell against which the needle subsequently touches.

The suspension was inoculated into the yolk using a tuberculin syringe and a No. 20 needle 1¼ inches in length. The amount of suspension injected varied from 0.1 to 0.6 cc.

All procedures were carried out aseptically. The hole in the shell was sealed with a vaseline-paraffin mixture. Eggs were incubated at 38°C.

We have had some early embryo deaths not due to contamination. Whether these are due to mechanical causes or some effect of the inoculum is not clear at this time.

The tumors available to us have not thus far produced as high percentage of "takes" as originally reported by Taylor.[2]

The ultimate embryo mortality is high. The causes will be considered in a separate report.

The embryos which die are small for their age.

The chicks which hatch do so late. They appear to be less vigorous than control chicks. The general tendency has been toward improvement after hatching. Some have appeared quite normal by the third day.

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* Aided by grants from the Medical Research Fund, Graduate School, University of Minnesota, and the Sivertsen Foundation for Cancer Research.

[1] Taylor, D. R., McAfee, M., and Taylor, A., *Cancer Research*, 1943, **3**, 542.

[2] Taylor, A., Thacker, J., and Pennington, D., *Science*, 1942, **96**, 342.

[3] Taylor, A., Hungate, R. E., and Taylor, D. R., *Cancer Research*, 1943, **3**, 537.

Nine chicks have hatched and lived; one died emerging from the shell. The living chicks were killed with ether and examined at 28, 48, or 72 hours after hatching.

The yolk sac is normally taken into the abdominal cavity prior to hatching where it occupies a large part of the cavity in the newly hatched chick and lies in intimate contact with the organs. It persists for more than 2 weeks. That part of the sac which enters the cavity last is normally slightly adherent to the body wall at the umbilical area.

The tumor-bearing yolk sac was found in the normal position. It was more than normally adherent to the umbilical area. Most of the tumors were found near the point of contact with the umbilical area, bulging into the yolk sac. In 2 cases they were scattered generally over the inner surface. In size they varied from less than 1 mm to about 10 mm. Larger masses were lobulated. Adjacent membranes were thickened. The tumors were usually firm, fleshy masses. In one case a cystic tumor was found.

In general appearance the tumors did not differ from those which we have examined in the late stages before hatching.

Of 9 living chicks from inoculated eggs, 7 showed gross tumors and 7 were confirmed on section.

Microscopically a majority of the tumors show alterations which suggest damage by the host.

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The Anti-Spirochetal Activity of Penicillin in Experimental Infections.

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1. *Activity of penicillin in experimental rabbit syphilis.* Mahoney, Arnold, and Harris[1] have reported on the anti-spirochetal activity of penicillin in rabbit syphilis. Definite spirocheticidal activity of penicillin *in vitro* tests has been reported more recently by Dunham and associates,[2] and by Eagle and Musselman.[3]

These findings form the experimental basis of the clinical results obtained with penicillin in human syphilis, as recently described by Mahoney and co-workers,[1] and by Bloomfield, Rantz, and Kirby.[4]

The purpose of our investigation was to establish the minimal healing and sterilizing doses for penicillin in experimental spirochetal infections. The results are presented in this report.

Experimental. The strain of rabbit syphilis used in our investigation was received from the Laboratories of the U. S. Public Health Service in 1940 and has since been transferred at 2-3-month intervals. Rabbits infected intratesticularly 8 weeks before treatment with this strain of *Treponema pallidum* showed orchitis of both testicles besides chancriform lesions containing large numbers of spirochetes. Treatment consisted of intravenous injections of a penicillin sodium salt produced in the Roche chemical research laboratories. Some material not suitable for clinical use was at our disposal. The potency of the different samples as determined in the cup test varied between 100 and 300 units/mg. The drug was administered from 4 to 6 times over a period of 48-72 hours. The first injection was usually given in the evening, the lesions were examined for spirochetes the next morning, and thereafter the injections were repeated in intervals of 3 to 18 hours.

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[1] Mahoney, J. F., Arnold, R. C., and Harris, A., *Am. J. Public Health*, 1943, **33**, 1387.

[2] Dunham, W. B., Hamre, D. M., McKee, C. M., and Rake, G., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 158; see also *J. Bacteriol.*, 1944, **47**, 22.

[3] Eagle, H., and Musselman, A. D., *J. Bacteriol.*, 1944, **47**, 22.

[4] Bloomfield, A. L., Rantz, L. A., and Kirby, W. M. M., *J. Am. Med. Assn.*, 1944, **124**, 627.

Three rabbits received 4 to 6 intravenous injections of single doses ranging from 33,000 to 47,000 units/kg. Eighteen hours after a single injection with this dose a striking decrease of the number of spirochetes was noticed in the lesions of 2 rabbits, and complete disappearance of spirochetes in the third rabbit. After the third injection no spirochetes could be found in any of the lesions. Definite regression of the orchitis and of the lesions was observed 4-5 days after the first treatment. The primary lesions were completely healed 11-16 days after treatment was started. A fourth rabbit was treated with the same single dose 5 times at 2-hour intervals (total dose 150,000 units/kg). On the following day only a few spirochetes could be found in repeated examinations of the lesions. On the second day after treatment, none could be found while regression of the lesions became manifest. One week later the testicle was completely healed.

Three rabbits received 4 intravenous injections with single doses of 7,500-10,000 units/kg over a period of 48-72 hours. Eighteen hours after the first injection of this dose, a decrease in the number of spirochetes was found in the lesions of one of the 3 rabbits. After the third injection, spirochetes disappeared completely in the lesions of all 3. Dissolution of the lesions was apparent 5-6 days after the first treatment, while after 10-16 days healing of the primary lesions was complete.

Two rabbits were injected 4 times with single doses of 3,800-4,300 units/kg. No decrease in the number of spirochetes was noticed 18 hours after this treatment. Spirochetes did not disappear from the lesions until after the third injection. Marked regression of the orchitis was recorded 5 days after the initial treatment. Healing of the primary lesions occurred within 10 days in the first rabbit, and within 15 days in the second. At the same time 6 untreated control rabbits still showed orchitis and lesions containing spirochetes.

The activity of lower doses has not yet been established; neither can the question of permanent sterilization be answered satisfactorily, since the results of lymphnode transfers are not yet available.

The observations indicate that penicillin exerts a very definite activity on testicular infections of rabbits with *Treponema pallidum.* They are comparable to those observed in human cases.

2. *Activity of penicillin in relapsing fever* (Borrelia novyi) *in mice.* The spirochetes of relapsing fever (*Borrelia novyi*) seem to be approximately as sensitive to penicillin as is *Treponema pallidum.*

Heilman and Herrell[5] found that a dose of 1,000 units was needed to prevent death as well as relapses in the majority of animals infected with *Borrelia novyi.* After administration of very high doses of penicillin, viz., 9,000 units per mouse, Augustin and his coworkers[6] observed recovery of mice infected with this parasite. According to Eagle and Magnuson,[7] the curative dose of penicillin in experimental infections of mice and rats with *Borrelia novyi* was 100,000 (50% cured animals) to 400,000 units (95% cured animals) per kg. The successfulness of treatment was ascertained by transfer of blood from treated to normal animals.

The purpose of our experimental work with *Borrelia novyi* in mice was to determine the minimal effective dose of penicillin in infections with the spirochetes of relapsing fever.

Experimental. The strain of *Borrelia novyi* used in these experiments was received from Dr. Malcolm H. Soule. Mice were infected intra-abdominally with a suspension of mouse blood containing 3 to 6 spirochetes per microscope field (dark field illumination). After 20 to 24 hours, spirochetes were present in the peripheral blood, generally 1 parasite per field. The mice were treated subcutaneously with penicillin using single doses ranging from 5,000 units/kg to 50,000 units/kg. In order to administer a high total dose (more than 50,000 units/kg), the treatment was given repeatedly in 3-hour intervals. Doses of

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[5] Heilman, D., and Herrell, W. E., *Proc. of the Staff Meetings of the Mayo Clinic*, 1943, **18**, 457.

[6] Augustin, D. L., Weinman, D., and McAllister, J., *Science*, 1944, **99**, 19.

[7] Eagle, H., and Magnuson, H. J., *J. Bacteriol.*, 1944, **47**, 21.

TABLE I.
Activity of Penicillin Na Salt on *Borrelia Novyi* in Mice.

Penicillin, units/kg	No. of Mice Treated	Blood cleared*	Relapses	Positive Reinfection
350,000†	2	2	0	0
200,000†	3	3	0	3
150,000†	3	3	1	0
100,000	3	3	0	—
50,000	17	17	9	2
25,000	6	6	1	0
12,500	5	1	1	0
5,000	3	0	—	—
Controls	10	0	6	0

* No spirochetes found by microscopic examination (dark field illumination) 20 hours after the treatment.

† Repeated treatment every 3 hours with single doses of 50,000 units/kg.

50,000 units/kg or less were given only once. Three to 4 weeks after treatment the mice were re-infected intra-abdominally with a heavy suspension of spirochetes. While we are aware of the fact that in spirochetal infections a positive re-infection is not absolute proof of previous successful sterilization, it might well provide some tentative information as to the curative effectiveness of antispirochetal agents. The results of the tests are given in Table I.

The minimal effective dose of penicillin in mice infected with *Borrelia novyi* was 25,000 units/kg, given subcutaneously one day after intra-abdominal infection; the spirochetes disappeared from peripheral blood within 4 hours after treatment. Relapses were observed only infrequently with doses up to 150,000 units/kg, and none occurred after treatment with doses ranging from 200,000 to 350,000 units/kg. Reinfection 3 to 4 weeks after the disappearance of parasites gave positive results in all mice treated with 200,000 units and in 2 out of 8 mice treated with 50,000 units. These results are in close agreement with the figures given by Eagle and Magnuson[7] and indicate that penicillin possesses considerable spirocheticidal activity. No definite statement can be made as yet as to the permanently sterilizing effect of penicillin.

Summary. 1. The single intravenous dose of penicillin sodium salt required to produce disappearance of *Treponema pallidum* in experimental rabbit syphilis is 33,000-47,000 units/kg. The same effect can be obtained with about 16,000 units/kg if the drug is administered repeatedly over a period of 72 hours in doses of approximately 4,000 units/kg. The latter dose was found sufficient to produce healing of primary lesions in 10-15 days.

2. The minimal effective dose of penicillin sodium salt in *Borrelia novyi* infections of mice was found to be 25,000 units/kg mouse; doses of 200,000 units/kg and more might be considered as curative.

3. No definite statement as to the sterilizing potency of penicillin in spirochetal infections can be made at the present moment.

Addendum.

The results of the lymphnode transfer of the syphilitic rabbits are now available. The lymphnodes of 4 rabbits treated with the highest doses of penicillin used, viz., 132,000-282,000 units/kg, transferred into testicles of healthy animals, produced a syphilitic infection, indicating that even the high doses of penicillin were not sufficient to sterilize the animals.

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Possible Role of Potassium in Pullet Disease.*

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So-called "pullet disease"[1,2] represents primarily an acute hepatonephrosis associated with degenerative changes in pancreas and voluntary muscles. It attacks chickens over 3 months of age during the summer, and occurs widely in the Northeast. Our laboratory diagnostic data on 1154 case lots for 1940-43 showed an average incidence of 22.7%, second in rank to the avian leukosis complex which was 29.9%. During 1943 the attack rate was particularly severe, 34.5% of 269 lots, causing severe drop in egg production and an average mortality of 5.2%. The entire cause is unknown since it has been impossible so far to reproduce the field syndrome with the virus isolated by Waller.[3,4] The characteristic explosiveness of outbreaks suggests a wide seeding of the etiologic agent acting synergistically with secondary factors. As empirical treatments, molasses in the feed, 1:2000 copper sulphate or 1:4000 potassium dichromate in the water[1] have been used, the first one being apparently effective, according to available field reports. Shortage of molasses for treatment during 1943 prompted studies on therapeutic substitutes.

Induced Renal Obstruction. In support of previous findings,[1] the 1943 data showed that out of 265 individual pullet disease affected birds, 37% were grossly and 67% were histologically affected with visceral gout (uric nephrosis). There are, therefore, reasons to believe that pullet disease is accompanied by renal damage. Experimental renal obstruction has been induced regularly in birds by parenteral injection of potassium dichromate[1] and recently of alloxan[5] but only irregularly by feeding of certain substances.[2] Oppenheimer[6] together with Kunkel[7] produced in chickens, fed an exclusive meat diet, articular gout which is rare under field conditions. Selye produced nephrosclerosis in chicks by repeated subcutaneous injection of desoxycorticosterone acetate,[8] watering with physiological salt solution, or both[9] and believed the experimental condition to resemble pullet disease.[10] Correll[11] in studying the relation of acid and alkaline-ash foods to vitamin D metabolism, observed sodium citrate and acetate to be toxic to chicks, causing the precipitation of an insoluble material in the body which phenomenon he termed the "salt effect." Feeding sodium citrate together with potassium citrate allowed for normal survival.

Experimental. Correll's[11] work appeared to offer an opportunity for studying protective factors against induced renal obstruction and was therefore repeated and extended. Pullorum clean day-old or 1-week-old R.I.R. or B.R. chicks were housed in sanitary batteries and fed simplified diet G7† and water *ad libitum*.

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* Supported in part by grants from the E. S. Farmers' Exchange and the Big "Y" Foundation.

[1] Jungherr, E., and Levine, J. M., *Am. J. Vet. Res.*, 1941, **2**, 261.

[2] Jungherr, E., in Biester, H. E., and Devries, L., *Diseases of Poultry*, Iowa State Coll. Press, 1943, 511.

[3] Waller, E. F., *Science*, 1943, **95**, 560.

[4] Waller, E. F., Tepper, A. E., Halpin, R. B., and Davis, H. A., *New Hampshire Agr. Exp. Sta. An. Rep.*, 1942, Bul. 345, 59.

[5] Jungherr, E., unpublished data, 1944.

[6] Oppenheimer, E. H., *Bull. Johns Hopkins Hosp.*, 1941, **68**, 190.

[7] Oppenheimer, E. H., and Kunkel, H. G., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 40.

[8] Selye, H., *Can. Med. Assn. J.*, 1942, **47**, 515.

[9] Selye, H., and Stone, H., Proc. Soc. Exp. Biol. and Med., 1943, **52**, 190.

[10] Selye, H., *J. Am. Vet. Med. Assn.*, 1943, **103**, 140.

[11] Correll, J. T., *J. Nutr.*, 1941, **21**, 515.

† Diet G7: Yellow corn meal 59.75, tech. casein 20, bagasse 10, brewer's yeast 5, gelatin 2, $Ca_3(PO_4)_2$ 2, NaCl 0.5, wheat germ oil 0.5, cod liver oil 0.25. Plus $MnSO_4$ 0.01%, pyridoxine 500/, calc. pantothenate 1400 γ/100 g feed.

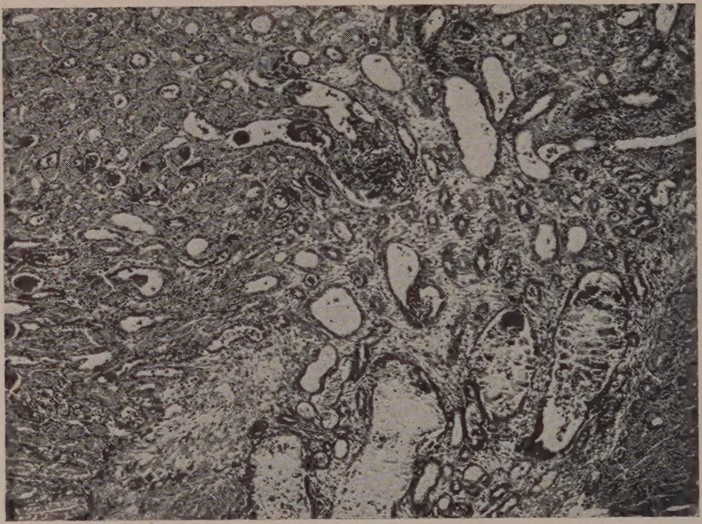

FIG. 1.
Kidney of chick fed toxic level of sodium citrate; marked tubular dilatation and cast formation. Azur II. 80×.

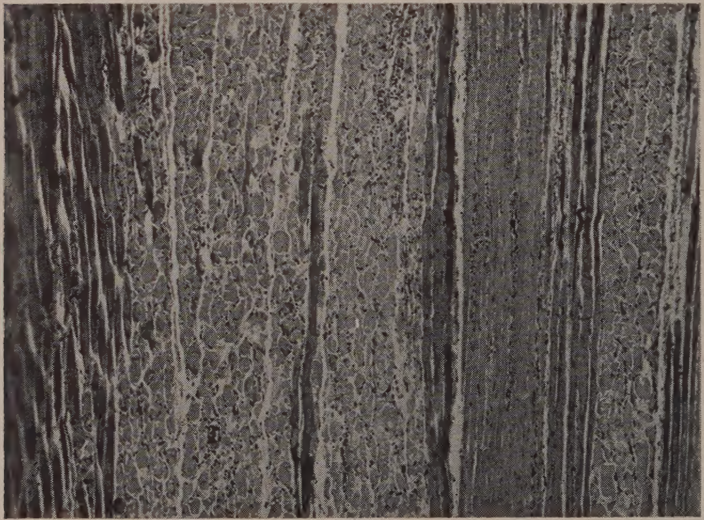

FIG. 2.
Breast muscle of clinically normal chick receiving excess of potassium chloride; the wide, lightly staining bands show Zenker's degeneration. H.E. 80×.

Various "toxic" and "protective" substances were added directly to the diet. The chicks were weighed once per week and, if surviving, sacrificed after 2 weeks of observation.

In general diet G7 was found to produce normal chicks with fair growth (average 89 g at 2 weeks). Renal obstruction induced by feeding high dosages of sodium citrate did not materially influence the growth rate of the survivors, but caused very high blood uric acid levels by the 3rd day which tended to fall off by the 8th day; these days corre-

TABLE I.
Mortality of Chicks 14 Days on Diet G7 Plus Various Supplements.

Serial item No.	Supplements				No. of chicks	Mortality %
	Toxic		Protective			
	Nature	%	Nature	%		
1	—		—		274	3.6
2	$NaHCO_3$	1.15	—		11	0
3	Trimethyl citrate	1.5	—		11	9.1
4	$C_6H_5O_7Na_3$	1	—		69	21.7
5	”	2	—		69	47.5
6	”	4	—		355	70.4
7	”	”	$KC_2H_3O_2$	1.97	11	9.1
8	”	”	K_2CO_3	1.39	11	36.3
9	”	”	$K_3C_6H_5O_7$	2.17	11	36.3
10	”	”	KI	3.34	12	83.3
11	”	”	KNO_3	2.03	10	0
12	”	”	$KMnO_4$	3.18	11	36.3
13	”	”	KCl	0.015-0.5	210	74.8
14	”	”	”	1	35	11.4
15	”	”	”	1.5	53	16
16	”	”	”	2	116	16.4
17	”	”	Muriate of Potash	1.57	42	7.1
18	”	”	Molasses in Feed	40	42	19
19	”	”	” ” Water	10	30	33.3
20	”	”	Glucose	10	46	71.7
21	”	”	$K_2Cr_2O_7$ in Water	0.05	54	72.2
22	”	”	$CuSO_4$ in Feed	0.05	54	81.5
23	”	”	” ” Water	0.05	30	50

sponded roughly to the period of highest mortality.

The so-called "salt effect" was found to be pathologically indistinguishable from the well-known picture of visceral gout or uric nephrosis. The kidneys were enlarged and showed fine whitish striation of the lobules; the ureters were distended due to urinary retention and the serous membranes of the body cavities were often covered with whitish urates. Microscopically, renal tissues showed extensive tubular dilatation and necrosis, together with formation of hyaline or heterophile casts (Fig. 1); occasionally typical "gout rosettes" were observed. In a few experimental lots high levels of "protective" potassium salts caused the appearance of asymptomatic whitish striation of the voluntary muscles (similar to those seen by Liebow *et al.* in mice[12]) which microscopically presented alternating bands of dystrophic and healthy muscle bundles (Fig. 2).

Results. To simplify presentation, the data for lots of chicks subjected to the same dietary regime but in various experiments have been combined and listed serially in Table I. A weighted average of the percent mortality of the lots was used as the main criterion, since major intercurrent diseases could be reasonably ruled out.

In *toxicity* experiments, the mortality on diet G7 without any supplements ranged from 0 to 27.3, with an average of 3.6%, and was considered nutritionally adequate (Item No. 1). Sodium bicarbonate shown by Witter[13] to cause renal obstruction in chicks if given in water, was found innocuous in the feed (No. 2); the citrate radical alone in the form of trimethyl citrate was likewise found to be nontoxic (No. 3). Sodium citrate proved toxic at levels of 1 and 2% (Nos. 4 and 5) but gave the most consistent results at the 4% level with an average of 70.4% mortality (range 28.6 to 91.9) (No. 6).

In all *protection* experiments 4% sodium citrate was used as the toxic principle. In preliminary trials several potassium salts such as potassium acetate (No. 7), carbonate (No. 8), citrate (No. 9), nitrate (No. 11), and potassium permanganate (No. 12) were found to afford varying degrees of protection. Potassium iodide (No. 10) seemed to be protective since none of the chicks showed the "gout" effect, but was toxic otherwise as indicated by retarded growth and high mortality. Since potassium, which is known to be essential for chicks[14] and animals in general,[15] seemed to be the protective principle a simple compound, potassium chloride, was tested on a larger scale. Six graded levels of from 0.015 to 0.5% (No. 13) failed to give protection, but 1 to 2% reduced the mortality by about ¾ (Nos. 14 to 16). Whether the 1% level was actually more protective than the higher level as it would appear from the data, must be rechecked on a larger number of birds.

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[12] Liebow, A. A., McFarland, W. J., and Tennant, R., *Yale J. Biol. and Med.*, 1941, **13**, 523.

[13] Witter, J. F., *Poultry Sci.*, 1936, **15**, 256.

To study the protective value of readily available substances and of empirical treatments for pullet disease, a sample of muriate of potash (Agri. grade 95-98%, Am. Potash and Chemical Corp.) as used in commercial fertilizers, was tested at the level of 1.57% and found to be protective (No. 17). Blackstrap molasses gave a significant measure of protection, more so in the feed (No. 18) than in the water (No. 19). The protective effect was apparently due to the known high potassium, and not the carbohydrate, content of molasses, since glucose alone (No. 20) afforded no protection. Potassium dichromate in water (No. 21) or copper sulphate either in the feed (No. 22) or water (No. 23) at the highest previously determined tolerated levels failed to give protection under the prevailing experimental conditions.

Pullet disease therapeutic trials. From a commercial flock of 3,500 chickens, aged 2½ to 17 months and known to be acutely affected with pullet disease, 30 clinically affected hens were brought to the laboratory and divided into two comparable lots. They were continued on the same brand of commercial feed except that 2% KCl was added to the mash feed of the one lot. Blood samples for uric acid were taken at the beginning of the experiment and again after 3 and 6 weeks. Chemical tests failed to show any significant differences between the groups, by analysis of variance. All birds were subjected to pathologic study. Only birds surviving for the 6-week experimental period were used in the computation of the data.

In the control lot of 15 birds, 3 died within one week and 1 within 2 weeks, all with typical gout. The average number of eggs laid per bird was 2.18. The average initial and final weight was 1943 and 2077 g, respectively. Pathologic examination showed gross enlargement of kidneys in 7 and microscopic residual kidney lesions in all of the 11 survivors.

In the KCl-fed lot of 15 birds, 6 died within less than 2 weeks and 1 in 5 weeks. The average number of eggs laid during the period of observation was 4.25. Initial and final weight figures were 2050 against 2040 g or about stationary for producing birds. Of the 8 survivors none showed gross and only 2 microscopic kidney lesions.

Although the number of birds used in this phase of the work was small, it would seem that those receiving the supplement of KCl were able to regain egg production faster and to combat kidney damage better than the individuals of the non-supplemented lot.

Summary. 1. According to laboratory records so-called pullet disease ranked second in pathologic importance in Connecticut chickens during the last 4 calendar years. 2. It was characterized in over ⅔ of the cases by kidney damage similar to visceral gout. 3. The "salt effect" resulting from feeding sodium citrate to chicks according to Correll, was found to correspond to experimental visceral gout. 4. The protective action of potassium salts against the "salt effect" was confirmed and shown to hold for simple compounds like potassium chloride or a good agricultural grade of muriate of potash at levels of 1 to 2% in the feed. 5. Molasses due to its potassium content was found to be protective, which rationalizes the corresponding empirical treatment for pullet disease. 6. In a small-scale experiment on spontaneous pullet disease cases, potassium chloride seemed helpful which suggests its use during molasses shortage.

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[15] Follis, R. H., *Arch. Path.*, 1942, **34**, 451.

14677

Shock Associated with Deep Muscle Burns.*

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Current views are still at variance as to whether surface burns lead to shock through drainage of fluid through the burned surface or through the formation of toxic substances in the damaged tissue. The question has also been raised whether any toxic substance that may be formed is not due solely to bacterial infection. In order to study this subject further we have observed the course of events after production of extensive aseptic deep muscle burns in the hind legs of anesthetized dogs by means of strong diathermy currents. In such preparations the volume of fluid which accumulates at the sites of the burns can be fairly exactly measured by immersion of the extremities.

Methods. Dogs, anesthetized with initial injections of 4 mg per kg of morphine and 175 mg per kg of sodium barbital, were used. The diathermy current from a Victor spark gap high frequency oscillator was led through moulded metal electrodes applied to the shaved skin with an intervening layer of electrocardiographic paste. The temperature of the deep structure, recorded with a Leeds and Northrup micromax by means of an iron-constantin needle thermocouple, was raised to 66° to 91°C during application of the diathermy current for 0.5 to 1.5 hours in the different experiments. About a third of the thigh muscles, or the thigh and calf muscles of one leg or the thigh and calf muscles of both legs were burned. The amount of fluid accumulating at the site of the burn was measured by recording the displacement of water when the legs were immersed separately in a tall cylindrical vessel until the symphysis pubis and iliac crest rested upon the mouth of the vessel. The opposite extremity was elevated about 40° above the horizontal and extended. The depth to which the skin entered the water was marked with quick drying paint. Two to four immersions were performed before burning and repeated again after death or, if the dog survived, at approximately 24 or 48 hours after burning.[1]

Results. In one group of experiments, 5 dogs were kept supine on animal boards during and following the burning in order that mean arterial pressure could be continuously recorded. These dogs were given small supplementary injections of sodium barbital from time to time. All died in 19 to 35 hours with a progressively declining arterial pressure and a rising hematocrit reading. Two of three controls observed simultaneously died, but in these the arterial pressure remained stable at the control level until within 0.5 and 1.5 hours before death. The results in these 8 dogs are summarized in Table I.

In a second group of experiments, 5 dogs were anesthetized and burned and were immediately placed in recovery cages. Water was withheld for the first 30 hours. Five control dogs were similarly treated except that they were not burned. All of the dogs survived 24 to 48 hours. Table II shows that the measured gain in volume of the legs of the burned dogs at this time was 21 to 31 ml/kg of dog. Since the dogs were all apparently in good condition, 9 of the 10 dogs were reanesthetized with 10 to 14 mg/kg of sodium pentobarbital and their mean arterial pressure recorded. The arterial pressure of the burned dogs ranged from 130 to 167 and that of the control dogs from 170 to 210. In order to see whether these dogs might be in a prodromal stage of shock[2] we studied their resistance to blood loss by comparing the volume of blood which had to be removed from the burned dogs with

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[1] For further details see Green, H. D., Dworkin, R. M., Antos, R. J., and Bergeron, G. A., *Am. J. Physiol.*, 1944, **142**, 494.

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TABLE I.

Trauma by Deep Muscle Burn—M.A.P. Recorded Continuously.

Traumatized dogs							Control dogs					
					Hematocrit reading						Hematocrit reading	
No.	Exp. No.	Survival time, hr	Measured limb vol. gain, ml/kg	MAP recorded for hr	Control	Max.	No.	Exp. No.	Survival time, hr	MAP recorded for hr	Control	Max.
1	KF-2	19	0	until died								
2	” 3	25.5	10	” ”	38	57						
3	” 4	35	11	” ”	53	59	1	KF-5	22	until died	51	64
4	” 7	23.5	18	” ”	53	63	2	” 6	17	” ”	55	69
5	” 8	25.5	3.5	” ”	52	69	3	” 9	Survived	27	48	48

TABLE II.

Trauma by Deep Muscle Burn—Dogs in Recovery Cages.

Traumatized dogs									Control dogs						
				A	B										
No.	Exp. No.	Time when bled, hr	MAP	Measured limb vol. gain, ml/kg	Bleeding vol., ml/kg	A + B, ml/kg	Vol. blood held out, ml/kg	Survival, hr	Exp. No.	Time when bled, hr	MAP_1	MAP_2	Bleeding vol., ml/kg	Vol. blood held out ml/kg	Survival, hr
1	KF-10	—	—	—	—	—	—	Killed 3rd day	KF-11	24	200		44	21	Survived
2	” 12	30	145	31	24	55	—	Killed end of bleeding	” 13	30	215	175	49	29.5	”
3	” 15	27	130	20.5	17	37.5	—	” ” ” ”	” 14	27	170	160	35	26	”
4	” 17	26	158	21.5	18.5	40	9	Killed 5th day	” 16	26	210	178	36	21.5	”
5	” 18	47	167	29	19.5	48.5	—	Killed end of bleeding	” 19	47	185	110	35	29	”

Footnotes for Tables I and II: Time measured from start of burning of trauma dog. Control dogs were anesthetized and observed simultaneously with the burned dogs. Limb volume gain and bleeding volume are expressed in ml/kg of body weight. MAP = mean arterial pressure. In Table II, MAP_1 is the pressure at the start of bleeding, MAP_2 is the pressure which existed when a volume of blood, equivalent to the volume of edema in the burned dogs, had been removed. Bleeding volume is the volume of blood which had to be removed in order to lower mean arterial pressure to, and to keep it at 50 mm Hg for 20 min. After establishing the "Bleeding Volume," part of the withdrawn blood was reinjected. The amount of blood which was not reinjected is indicated in the column labeled "Vol. of Blood Held Out." It will be noted that this is approximately the volume of the edema in the corresponding burned dog.

that which had to be removed from the control dogs in order to reduce their arterial pressures to 50 mm Hg. The results, summarized in Table II, show that the volume of blood which could be removed from the burned dogs was significantly lower than that of the control dogs. The sum of the local fluid accumulation in the burned legs plus the bleeding volume was in every case, however, equal to or greater than the bleeding volume of the corresponding control.

Because of the difficulty in caring for dogs with such severely burned legs, three of the burned dogs were killed immediately after the bleeding, and the remaining two were sacrificed on the third and fifth days. Only part of the withdrawn blood was reinjected into the controls, 21 to 29.5 ml/kg of body weight being withheld. All survived indefinitely.

Conclusions. It was not found possible to produce a progressive decline in arterial pressure, leading to death in lightly anesthetized dogs by means of deep muscle burns alone. A prodromal state of shock was produced, however, since the resistance of these dogs to bleeding was significantly less than that of control dogs similarly anesthetized but not burned. Since the volume of blood which had to be removed from the burned dogs in order to lower their mean arterial pressure to 50 mm Hg, plus the volume of edema in the legs, was as large as or larger in each case than the bleeding volume of the corresponding control dogs, it is concluded that the accumulation of fluid at the site of the burn is the principal factor responsible for the prodromal stage of shock in the burned dogs. Association of the extensive muscle destruction with restriction of movement and with prolonged moderately deep anesthesia converts the prodromal stage into outspoken shock with death of the dog even when the accumulation of fluid at the site of the burn is quite small.

14678 P

Functional Polarization in Retinal Development and Its Reestablishment in Regenerating Retinæ of Rotated Grafted Eyes.*

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The retina of adult salamander eyes always degenerates and is replaced by regeneration from surviving peripheral cells when the eye is grafted,[1,2,3] even repeatedly,[4] or when its blood supply is temporarily interrupted.[5] If the optic nerve is cut without interfering with the blood supply the retina survives and the nerve immediately regenerates.[5] This offers unusual opportunity to test the functional quadrants of the retina.

When Sperry first reported[6] reversed visuomotor responses in salamanders after optic nerve regeneration in eyes rotated *in situ*, I examined the effects of various types of rotation on vision in 100 grafted adult *Triturus viridescens* eyes in which a new functioning retina could be tested.

Normal visuomotor responses in salamanders are demonstrated when the animal moves toward, snaps at and follows a lure approaching any of the 4 quadrants in the field of vision. The animal also automatically moves

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* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University School of Medicine.

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[2] Stone, L. S., and Ellison, F. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 181.

[3] Stone, L. S., and Cole, C. H., *Yale J. Biol. and Med.*, 1943, **15**, 735.

[4] Stone, L. S., and Farthing, T. E., *J. Exp. Zool.*, 1942, **91**, 265.

[5] Stone, L. S., and Chace, R. R., *Anat. Rec.*, 1941, **79**, 333.

[6] Sperry, R. W. (Abst.), *Anat. Rec.*, 1942, **84**, 20.

head and body in the same direction with a rotating black and white drum only if it passes posteroanteriorly through the field of vision of an eye[7,8,9] (clockwise for a left eye and counterclockwise for a right eye). These compensatory movements are not elicited when the drum moves anteroposteriorly through the vision field of either eye.

The right or left eye was rotated 180° *in situ* and fixed in place after cutting conjunctival and muscular attachments. The optic nerve and blood supply was left intact to preserve the original retina. The results were the same as in similar experiments by Sperry. All quadrants of the original intact retina were functionally reversed. After removing the normal eye on the opposite side the animals tended to swim and walk in circles toward the eyeless side. The head usually proceeded in the same direction when the animal came to rest. To a rotating drum the head and body movements were elicited only when the drum passed from the original posterior to the original anterior pole, but they were reversed—a direction opposite to that of the moving field. When the lure was brought into the fields of each of the 4 retinal quadrants the animal reversed its reactions and sought the lure in the opposite direction. After the eye was rotated back to normal position again, normal visuomotor reactions were restored.

When the eye was excised and reimplanted upside down (180° rotation of all quadrants) return of vision through the new retina showed complete reversal of all quadrants as in the case of rotated eyes retaining their original retinæ. The results were the same as those reported by Sperry in rotated eyes whose optic nerves were later cut and allowed to regenerate.

When the left or right eye was excised and grafted to the opposite side so that dorsal and ventral quadrants were normally oriented, the anterior and posterior poles were then reversed (180° rotation). After vision returned in the new retina the swimming, head movements, and reversed reactions to the rotating drum were the same as in the case of rotated eyes mentioned above. Reactions to the lure were completely reversed and abnormal only in the rotated anterior and posterior quadrants.

When the right or left eye was excised and grafted on the opposite side, upside down, the dorsal and ventral quadrants were reversed, leaving the anterior and posterior quadrants normally oriented. Head and body movements, swimming, and reactions to the rotating drum were the same as in animals with one normal eye, since the anterior and posterior quadrants were not reversed. To the lure, reactions were reversed only in the rotated quadrants, the dorsal and ventral.

These experiments indicate that the functional pattern is different in each of the 4 retinal quadrants and that each is reestablished in the regenerated retina ond properly connected with the optic tectum to function according to its orientation.

In an attempt to determine the stage in development at which the retina becomes functionally polarized, the optic vesicle and early cup were excised, rotated 180° and reimplanted in embryos from the closure of the neural folds to late tail bud in *Amblystoma punctatum*. Vision tests during the larval and adult life of these hosts demonstrated functionally normal eyes. When the eye was excised, rotated 180°, and reimplanted at the time retinal layers were beginning to differentiate, vision tests later showed complete reversal of all retinal quadrants. Experiments on intermediate stages are now under way to determine the exact developmental period at which the retinal quadrants become functionally established. A 400-foot colored motion picture film has been made recording these experiments.

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14679

Effect of Ligation of Pancreatic Ducts on the Serum Phosphatase.

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The following experiments deal with the changes in the phosphatase of the serum of dogs in which the pancreatic ducts have been ligated and cut. That the blood diastase may be influenced by ligation of the pancreatic ducts has been shown by Pratt and his co-workers. Golden, Sieracki, Handelsman, and Pratt[1] have found that the permanent separation of the pancreas from the duodenum through tying the pancreatic ducts resulted in a persistent increase of the diastase in the blood in 3 out of 4 dogs. Bodansky and Jaffé[2] ligated the common bile duct of a dog and found a progressive elevation in the serum phosphatase paralleling the rise in the serum-bilirubin. These ligation experiments have been confirmed by Armstrong, King, and Harris[3] in 19 dogs. After releasing the obstruction in 2 dogs these authors noted a return to normal in the phosphatase and bilirubin values.

Alkaline phosphatase has been shown to be present in pancreatic tissue. In the course of our studies on dogs with a pancreatic fistula we have found alkaline phosphatase also in the pancreatic juice ranging between 6.0 and 15.5 Bodansky units.

Regarding the above mentioned experiments of Pratt and his co-workers, of Bodansky and Jaffé, and of Armstrong, King, and Harris, we became interested in the question whether the serum phosphatase is influenced by the separation of the pancreatic gland from the duodenum through ligation of its ducts. Our findings are based on observations on 10 dogs.

Determination of alkaline serum phosphatase with Bodansky's method was continued for days and even months when the animals survived. The pancreatic ducts were dissected out under nembutal anesthesia, ligatures were applied and the ducts were then cut. The dogs were supplied with food and water each day, and no therapeutic measures were applied except that in the last 3 operated dogs we put sulfathiazole powder in the abdominal cave in order to improve the process of healing after the operation. Blood was withdrawn from the heart by puncture prior to the operation and at different days succeeding the operation for estimation of phosphatase.

The results of our examinations may be found in Table I.

We have determined the alkaline serum phosphatase 20 times in 10 dogs before the operation. The average amount was 1.94 Bodansky units. The deviation was not significant, the highest value being 3.3 units, the lowest value 1.4 units. The day following the operation the majority of the dogs had recovered satisfactorily and they were taking food. The alkaline serum phosphatase began to rise, but only in dog 8 was there a definite and marked increase from 1.4 units to 12.4 units within the first 24 hours. The serum phosphatase rose to progressively higher values each day following the ligation, reaching its peak between the fourth and the tenth day after the operation. A value 6 to 8 times higher than the initial value was found in every dog where the blood could be examined within this time. Three dogs (No. 2, 5, and 13) showed only a very moderate increase of the serum phosphatase, but these dogs died within 24 to 72 hours after the operation had been performed. Between the 10th and the 20th day after the ligation the values tended to decrease but returned to the initial value in only one dog.

Acid serum phosphatase was also determined before and after the closure of the pan-

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[2] Bodansky, A., and Jaffé, H. L., *Am. J. M. Sc.*, 1936, **192**, 527.

[3] Armstrong, A. R., King, E. J., and Harris, R. I., *Canad. M. A. J.*, 1934, **31**, 14.

TABLE I.
Alkaline Serum Phosphatase Before and After Ligation of the Pancreatic Ducts.

Dog No.	Alkaline serum phosphatase in Bodansky units before operation	Alkaline serum phosphatase in Bodansky units after operation					
		1	2	3	4 to10	11 to 20	Beyond 20th day
2	2.8	3.43	3.4				
3	2.2 1.95				10.5 (6)*	8.8 (11)	
5	3.3 1.7 1.5 1.9	4.94					
6†	2.0 2.5 2.2				12.5 (4) 13.8 (7)	2.13 (12) 1.55 (18)	3.5 (44) 1.75 (55)
8	1.4 1.65	12.4		10.8			
9	1.8 1.6 1.4	5.4	12.4				
13	2.2	4.8	7.2				
14	1.8 1.6	2.2			14.2 (8)	10.8 (11)	
15	1.86		5.93		14.75 (7)	10.42 (18)	4.67 (22) 4.90 (45) 3.27 (70)
17	1.8 2.08	2.26			16.4 (8)	13.55 (11)	

*The number in parenthesis means the exact day after operation.
†Autopsy showed that occlusion of all ducts has not been completely accomplished.

creatic ducts. The figures before the operation ranged between 0.6 and 4.0 Armstrong units. No influence of the operation on the level of acid serum phosphatase could be found.

Ligation of the pancreatic ducts in dogs has been shown to result in a marked increase in alkaline serum phosphatase, the values reaching 6 to 8 times the initial amount in 4 to 10 days. As the liver of 3 of our dogs has been examined histologically and no signs of liver damage have been found, the increase in the values in alkaline serum phosphatase appears to be due to the occlusion of the pancreatic ducts and not to liver damage which may have developed in connection with the operation.

The results of our experiments may become significant in evaluating observations on patients with pancreatic disorders. All our cases with carcinoma of the pancreas disclosed markedly elevated values for alkaline serum phosphatase. Since all of them had jaundice the elevated figures could be explained by obstructive jaundice. But in 2 cases of steatorrhea and low volumes for pancreatic juice and low bicarbonate values after injection of secretin we have also found elevated figures for alkaline serum phosphatase. Before a definite conclusion can be reached as to the value of the estimation of alkaline serum phosphatase in the diagnosis of pancreatic disease the experiments need to be supplemented by a larger number of observations on human cases of obstruction of the pancreatic duct.

Summary. Ligation of the pancreatic ducts in dogs resulted in the increase of the alkaline serum phosphatase in 7 out of 10 animals. The values reach their peak between the 4th and the 10th day after the operation and remain high between the 10th and 20th day after the occlusion of the ducts. They are 6 to 8 times higher than the values before the operation. The 3 dogs which showed a moderate increase of alkaline serum phosphatase died within 24 to 72 hours after the operation had been done.

Acid serum phosphatase is not influenced by the ligation of the pancreatic ducts.

14680 P

Diagnosis of Leishmaniasis by Slide Agglutination.*

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Numerous serological studies on leishmania infections have provided information on the following reactions: Agglutination,[1-4] complement fixation,[4,5] reversed complement fixation,[6] and intradermal tests.[4,7-11] Moreover, complement fixation,[12,13] precipitin,[14] agglutination,[15] and lysis,[16,17] as well as slide agglutination,[17] have been obtained in *Trypanosoma cruzi* infection. In the present study slide agglutination in leishmaniasis as well as the relationship of homologous and heterologous immune sera to this reaction have been tested.

Antigens for immunization were prepared according to the writer's method.[17] Two rabbits each were immunized with killed *Leishmania brasiliensis, L. donovani, L. tropica*, and *T. cruzi* by intravenous injection 3 times a week. The initial dose of 0.5 cc was increased by 0.1 cc to 1 cc, which amount was then maintained until a total of ten injections had been given. A week later each animal was bled from the marginal ear vein and the titer of agglutinins determined. Immunization with living cultures 3 times a week was then carried out. The initial dose of ¼ culture slant 4 to 6 days old was increased to 1½ slants, with a total inoculation of 5½ slants. A week later, the animals were again bled and the agglutinin titers were determined.

Slide agglutination antigen and "H" antigen were prepared as previously described;[17] "O" antigen, by adding gradually with constant shaking 70% alcohol to a saline suspension of organisms until the final concentration of alcohol was 50%. The suspensions were left overnight at room temperature, washed 3 times in physiological saline solution and then standardized. Slide and tube agglutination tests were performed according to approved technics.[17]

Results—Table I shows the titers of slide agglutination, and of "H" and "O" agglutination of rabbits immunized with killed and living cultures of the organisms tested.

Conclusions. Rapid diagnosis of visceral, cutaneous, and mucocutaneous leishmaniasis may be made by slide agglutination. "H" and "O" agglutinogens are present in all species tested. The homologous organism is agglutinated to a high titer, but co-agglutinins

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TABLE I.
Agglutination Test in Rabbits.

Rabbits immunized with	*L. brasiliensis*			*L. donovani*			*L. tropica*			*T. cruzi*		
	Slide	Antigen “H”	“O”	Slide	Antigen “H”	“O”	Slide	Antigen “H”	“O”	Slide	Antigen “H”	“O”
L. brasiliensis												
1. Killed	1:640			1:320			1:80			1:80		
Living	1:640	1:800	1:200	1:320	1:400	1:200	1:80	1:400	1:200	1:80	1:200	1:100
2. Killed	1:320			1:80			1:40					
Living	1:1280	1:1600	1:400	1:320	1:400	1:200	1:80	1:400	1:200	1:20	1:200	Neg.
L. donovani												
3. Killed	1:20			1:640			1:20			1:2		
Living	1:40	1:100	1:100	1:2560	1:12800†	1:1600	1:20	1:200	1:200	1:40	1:400	1:100
4. Killed												
Living	1:40	1:100	1:200	1:320	1:6400	1:1600	1:20	1:100	1:200	1:20	1:200	1:100
L. tropica												
5. Killed	1:160			1:2			1:160			1:40		
Living	1:160	1:400	1:400	1:20	1:100	1:200	1:640	1:800	1:400	1:80	1:200	Neg.
6. Killed	1:640			1:2			1:20			1:20		
Living	1:640	1:800	1:200	1:40	1:200	1:200	1:640	1:800	1:400	1:40	1:400	1:100
T. cruzi												
7. Killed	1:40			1:2			1:40			1:160		
Living	1:40	1:100	1:100	1:20	1:200	1:200	1:20	1:200	1:200	1:1280	1:3200	1:200
8. Killed	1:40			1:160			1:20			1:320		
Living	1:40	1:400	1:200	1:320	1:400	1:400	1:40	1:200	1:200	1:1280	1:3200	1:200

are present in low titer to the heterologous organisms. *L. donovani* and *T. cruzi*, responsible for systemic infections, produce higher titers than *L. tropica* and *L. brasiliensis*, which have only cutaneous or mucocutaneous manifestations.

14681

Effect of Adrenocorticotrophic Hormone on Leucocyte Picture of Normal Rats and Dogs.*

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Experimental evidence is available which indicates that stimulation of the adrenal cortex by injection of adrenocorticotrophic hormone (ACTH) produces a marked decrease in the weight of the thymus and lymph nodes in the mouse[1] and in the rat.[2] Subsequent studies by Dougherty and White on the white blood cells in the mouse and rat[3] and dog[4] after such treatment have demonstrated a typical change in the leucocyte picture characterized by transient leukopenia with an absolute lymphopenia and an absolute polymorphonuclear leucocytosis. The reaction was thought to be specific in that the effects were not observed in the treated adrenalectomized animal, and were not duplicated by the injection of control substances. The same response, moreover, was produced by the injection of adrenal cortical extract into normal and adrenalectomized mice.

Certain of the above findings studied in this laboratory have been confirmed. Acute and chronic injections of purified ACTH in normal rats of the Long-Evans strain have resulted in the production of an absolute lymphopenia and polymorphonucleosis. A typical experiment is presented. Purified ACTH[5] was injected into normal 26-day-old male rats in doses IP (intraperitoneal) of 1 mg every 4 hours. Four hours after the second injection, leucocyte counts and differential counts were made from tail blood samples. The findings are presented in Table I. It is apparent that ACTH treatment is followed by a mild leukopenia with a definite absolute change in the ratio of polymorphonuclear to mononuclear cells.

Injections of ACTH were carried out on fasted adult normal dogs to ascertain the type of reaction which might be obtained in this animal form. Purified sheep ACTH was injected IP in dosages of from 10 to 100 mg. Duplicate white cell counts and differentials were made at 2-3-hour intervals until counts and differential percentages returned to normal control levels. Specimens of blood were obtained from the femoral artery in unanesthetized animals. Control experiments were carried out using similar amounts of purified lactogenic hormone,[6] boiled (inactivated) interstitial cell stimulating hormone[7] (ICSH), and boiled inactive pituitary gland residue. The experiments are summarized below. Peak leucocytoses were noted in all animals within a period of 5-10 hours. The white cell counts returned to normal within

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* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation, New York City.

[1] Dougherty, T. F., and White, A., Proc. Soc. Exp. Biol. and Med., 1943, **53**, 132.

[2] Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 135.

[3] Dougherty, T. F., and White, A., *Science*, 1943, **98**, 367.

[4] White, A., personal communication.

[5] Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

[6] Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, 1942, **146**, 627.

[7] Li, C. H., Simpson, M. E., and Evans, H. M., *Endocrin.*, 1940, **27**, 803.

TABLE I.
Effect of ACTH Injections on Leucocyte Picture in Normal Male Rats.

Exp.	No. of animals	Avg B.W. g	White cells per cmm	% Polymorphonuclear cells	% Mononuclear cells
ACTH treated	6	71	5800 (3850-7600)*	69 (63-79)	31 (21-37)
Control untreated	6	71	6875 (5400-8400)	24 (17-31)	76 (69-83)

* Figures in parentheses indicate ranges in the groups.

TABLE II.
Effect of Injection of ACTH on White Blood Cell Count in Normal Dogs.

Dog No.	Treatment (I.P. injection)		White cell/cmm		
			Initial	Peak	% Increase
1	ACTH 100 mg		12250	37075	
2	” 100 ”		8950	29425	
3	” 100 ”		10550	29400	
4	” 10 ”		15500	36700	
Avg					286
5	Boiled ICSH	100 mg	11375	19875	
6	” ”	50 ”	9074	19800	
7	Lactogenic	100 ”	15400	28400	
8	Inactive boiled residue	100 ”	24000	47300	
Avg					193

TABLE III.
Effect of Subcutaneous Injection of ACTH on Leucocyte Picture in the Dog.

Dog No.	Treatment	White cell count		Total lymphocyte count	
		Initial	After 6 hr	Initial	After 6 hr
1	ACTH 10 mg	11900	21025	2618	1051
2	” 10 ”	12050	13025	964	390
3	” 10 ”	9325	13625	1771	408
4	Lactogenic 10 mg	19500	22500	3120	3375
5	Boiled ICSH 10 ”	12000	13250	2040	1987

24 hours after injection. The differential cell counts in the ACTH and the control treated animals did not differ significantly, there being an increase of polymorphonuclear leucocytes to approximately 90% in each group. The curves of the leucocyte counts were of the same character in ACTH and control treated animals, the only difference being apparent in the greater quantitative increase in leucocytes in the ACTH treated group.

Further experiments were carried out in dogs employing subcutaneous injections of purified ACTH and other anterior lobe preparations. In these experiments, 10 mg of the hormone preparation in isotonic saline were injected beneath the skin of the groin. Blood samples were taken from the femoral artery. The data obtained are presented in Table III.

The total lymphocyte counts in the ACTH treated animals returned to the original level 24 hours after injection of the hormone preparation. The lymphopenia here demonstrated on subcutaneous injection of ACTH in the dog confirms the findings of Dougherty and White[4] for the same animal. It is probable that the marked polymorphonuclear leucocytosis evoked by intraperitoneal injection in the dog obscures the specific effect on the lymphocyte picture which is seen on subcutaneous injection in the absence of a clearcut leucocytosis.

The reported experiments on the effect of ACTH on the various tissues (thymus, lymph nodes, and white blood cells) suggest the employment of ACTH or adrenal cortical substances experimentally and clinically in the treatment of conditions associated with overgrowth or neoplastic processes in the lymphoid tissues. Experiments in the transplantation of neoplastic tissues in rats either adrenalectomized or treated with ACTH or adrenal cortical substances by Murphy and Sturm[8] give further support to the hypothesis that these tissues are under the hormonal influence of the adrenal cortex.

Summary. IP injection of adrenocorticotrophic hormone (ACTH) in the rat produces a transient leukopenia with decrease in number of lymphocytes and increase in the number of circulating neutrophils. Intraperitoneal injection of ACTH in the normal dog produces a marked neutrophilic leucocytosis which is qualitatively similar to but quantitatively greater than that produced by injection of other anterior lobe substances. Subcutaneous injection of ACTH in the dog produces a lymphopenia which is similar to that occurring in the rat after treatment with ACTH.

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[8] Murphy, J. B., and Sturm, E., *Science*, 1943, **98**, 568; 1944, **99**, 303.

14682

Gravity Shock in Rabbits. III. Changes in Distribution of Phosphate and Chloride in Blood and Urine.

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The significant chemical changes occurring in the blood of rabbits suspended by their ears until unconsciousness occurs are: decreased blood pH, CO_2, venous O_2, increased blood lactate, pyruvate, plasma potassium, inorganic phosphate and non-protein nitrogen. Urine flow falls to zero or nearly so. The changes are considered to be the result of hypoxia caused by peripheral circulatory deficiency.[1,2] To determine in part the source of the phosphate and whether or not a change in the chloride ratio occurs, studies have been made on the distribution of phosphorus and chloride in cells and plasma, and on the excretion of urinary phosphate in rabbits before, during, and recovering from gravity shock.

Methods. The animals used and the methods of producing shock, of withdrawing blood samples, of measuring pH, hematocrit and chloride content have been previously described.[2] Inorganic and acid-soluble phosphorus were determined by the Fiske and Subarrow method as modified by Guest and Rapoport.[3] Samples of blood were drawn just before suspension, just after unconsciousness, and from one to 2 hours after the animals had been returned to the horizontal position.

Results. The hematocrit during shock did not change consistently, but during recovery always decreased (Table I). Whether or not the rabbits had been fed preceding the experiment, the plasma and red cell inorganic phosphorus increased during shock, and was roughly correlated with the decrease in blood pH. The organic acid-soluble phosphorus in plasma usually increased during shock but in the cells it fluctuated around the control values. During recovery the plasma phosphorus compounds and the cell inorganic phosphorus tended to shift back towards control values, but the cell organic acid-soluble phosphorus increased significantly above control levels. Evidently the added inorganic phosphorus was not derived from food in the

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* For assistance in collecting the data on urine flow and urine phosphate determinations, we are indebted to Martin J. Jarvis.

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[2] Cole, W. H., Allison, J. B., Murray, T. J., Boyden, A. A., Anderson, J. A., and Leathem, J. H., *Am. J. Physiol.*, 1944, **141**, 165.

[3] Guest, G. M., and Rapoport, S., *J. Biol. Chem.*, 1938, **124**, 599.

TABLE I.
The Hematocrit and pH of the Blood, and the Distribution of Inorganic and Organic Acid-soluble Phosphorus in Red Blood Cells and Plasma of Rabbits Before, During and After Gravity Shock. Animals numbered 1, 2, and 5 died in shock. Animals which received no food 24 hours previous to gravity shock are indicated by an asterisk before the number in the first column.

Animal No.	State	pH	% red blood cells	Phosphorus—mg/100 ml			
				Plasma		Red blood cells	
				Inorg.	Organic acid-soluble	Inorg.	Organic acid-soluble
1	Control	—†	37.0	5.6	0.7	2.77	86.2
	Shock	6.68	37.1	14.3	2.80	6.41	90.3
2	Control	—	41.8	7.15	0.85	2.24	93.4
	Shock	6.58	45.2	24.6	4.20	13.7	88.0
3	Control	—	42.6	3.30	—	2.34	81.3
	Shock	7.23	41.8	5.20	0.68	2.67	80.3
	Recovery	7.39	36.1	3.90	0.90	1.0	91.4
*4	Control	—	42.5	5.98	0.42	3.03	89.8
	Shock	7.25	43.0	8.00	8.60	5.44	90.4
	Recovery	—	33.3	9.15	0.35	3.45	98.4
*5	Control	—	51.1	5.68	0.12	2.64	75.0
	Shock	6.85	47.7	16.40	0.0	8.63	94.9
*6	Control	—	41.4	6.42	0.78	2.34	70.8
	Shock	7.20	40.3	11.63	0.40	8.84	76.3
	Recovery	7.31	27.8	9.22	0.58	5.18	79.1
*7	Control	—	41.2	6.12	0	2.57	74.1
	Shock	7.15	42.8	12.20	2.6	8.41	68.7
	Recovery	7.35	30.8	9.13	1.77	3.28	85.0

† The control pH was assumed to be 7.33 which was the average of determinations on 28 different control animals in a previous series.[2]

TABLE II.
The Hematocrit and pH of the Blood, and the Distribution of Chloride Ions Between Red Blood Cells (c) and Plasma (p) Before (Control) and During Gravity Shock (Shock).

Animal No.	State	pH	% red cells	Plasma chloride meq./l	Cell chloride meq./l	Cl_c/Cl_p
1	Control	—	37.2	109.0	74.0	0.68
	Shock	7.06	36.2	110.0	79.6	0.72
2	Control	—	47.5	110.8	61.7	0.56
	Shock	6.72	51.8	95.7	79.9	0.83
3*	Control	—	42.6	107.2	54.4	0.51
	Shock	7.23	41.8	104.4	60.6	0.58
	Recovery	7.39	36.1	108.6	59.3	0.55
4	Control	—	47.5	110.8	59.8	0.53
	Shock	6.98	46.1	101.8	61.2	0.60
5	Control	—	41.6	114.9	50.9	0.44
	Shock	6.90	45.2	109.8	89.5	0.82

* The same animal as No. 3 in Table I.

alimentary canal nor from organic acid-soluble phosphorus in the red cells.

The plasma chloride did not change significantly or decreased slightly during shock,

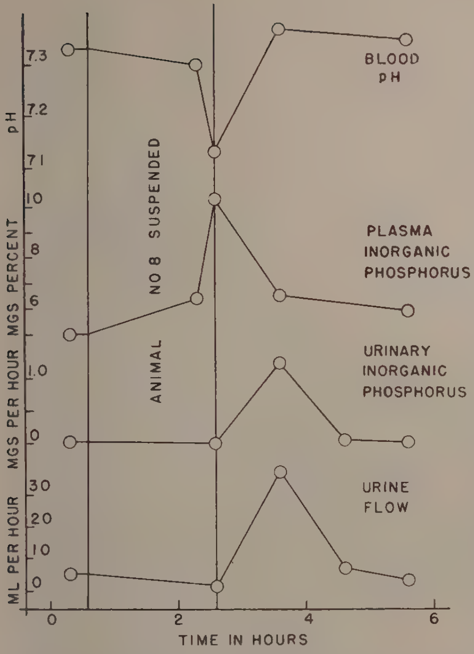

Fig. 1.

Changes in blood pH, concentration of plasma inorganic phosphorus, urine flow and total inorganic phosphorus excreted in urine per hour in a rabbit suspended for 2 hours and then returned to horizontal position. Period of suspension is indicated by the 2 vertical lines.

whereas the cell chloride definitely increased (Table II). The net result was always an increased ratio of cell chloride to plasma chloride. Since water in the cells and plasma was not determined, it cannot be decided whether the altered ratio was caused only by a chloride shift. Increase in the ratio was greater in the animals with a blood pH of 6.9 or less.

The effects of gravity shock and of recovery processes on urine flow and excretion of inorganic phosphorus as correlated with blood pH and plasma inorganic phosphorus are illustrated in Fig. 1. Four other animals gave the same kind of results, although in the majority of them decrease in plasma inorganic phosphorus and increase in urine flow during recovery were not as large as shown in No. 8. The data support the concepts that oliguria or anuria contribute to the development of the metabolic conditions associated with shock and that increased kidney function aids in the recovery from those conditions.

Discussion. It is assumed that the phosphatemia of gravity shock results from the breakdown of creatine and other organic phosphates in the tissues during stagnant hypoxia. The ratio between inorganic and organic phosphorus is thus increased. The simultaneous increases in blood lactate and pyruvate, so that the lactate-pyruvate ratio is increased, reflects the depletion of glycogen stores during hypoxia. Prolongation of that state results in depletion of adenyl pyrophosphate and phosphorylated enzymes, leading to a breakdown of normal metabolism. The large increase in plasma inorganic phosphate thus produced tends to reverse by mass action the liberation of inorganic phosphorus from the cell organic phosphates which is favored by the decreased blood pH.[4] The increased phosphate concentration in the cells probably replaces part of the bicarbonate which decreases because the pH and the CO_2 content of whole blood decreases.[2] The new cell-plasma equilibrium is reflected by the increased chloride ratio. Conversion of inorganic into organic phosphorus is favored during recovery as the pH returns to normal, accompanied by increased urinary excretion of inorganic phosphate, so that the phosphatemia disappears.

Summary. The increased inorganic phosphate in plasma during gravity shock does not originate from food in the digestive tract nor from the organic acid-soluble phosphorus in the red cells. The chloride ratio increases during gravity shock. During recovery diuresis and phosphaturia occur, while the pH, plasma phosphate, and chloride ratio rapidly return to control values. If those five processes do not occur the animal will die.

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[4] Guest, G. M., and Rapoport, S., *J. Lab. and Clin. Med.*, 1940-41, **26**, 190.

14683

The Sudden Death of Mice Injected Intraorbitally with Minute Quantities of Sesame Oil.*

Frank H. J. Figge.

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In the course of an experiment designed to test the sensitivity of the harderian gland to induction of tumors, about 50 mice were injected with methylcholanthrene in 0.05 cc of sesame oil. The oil was injected intraorbitally into or near the harderian gland. The mice were lightly anesthetized with nembutal and under these conditions no immediate effect of the injection was noticed during the first 10-30 seconds after injection. As fast as the mice were injected, they were placed in a container on a shelf to recuperate from the anesthetic. During a slight interruption in the injection procedure, it was noted that all 45 of the injected mice were dead or dying. It was thought that the anesthetic might be responsible, but some of the anesthetized mice were allowed to recuperate and showed no ill effects. Unanesthetized mice were then injected with 0.05 cc of plain sesame oil. They all died with characteristic symptoms. About 10 seconds after injection they began to breathe rapidly; some of them then went into convulsions and stopped breathing 1-2 minutes after injection. Others sprang into the air and respiration ceased within 1 minute.

Various amounts of oil were injected intraorbitally into unanesthetized mice to determine the quantity necessary to produce the reaction. When water solutions were injected intraorbitally, some animals died, but the results were inconsistent. As little as 0.025 cc of any viscous oil always produced death, but the mice receiving 0.02 cc of oil usually survived. Those which did survive such minute amounts showed severe respiratory symptoms and were lethargic for several hours. Mice of both sexes and various ages all respond in a similar manner. No differences could be detected in the response of mice of mammary cancer susceptible strains (C3H and A) and mice from cancer resistant strains (C57, JK, I).

When the oil-injected mice were autopsied as soon as respiration ceased, the atria were observed to be fibrillating, but the ventricles were in diastole. It was thought that this reaction was related to the oculo-cardiac reflex. To test this hypothesis, mice were injected intraperitoneally with 0.05, 0.1, 0.3 mg of atropine sulfate. Fifteen to 30 minutes later, the mice were injected intraorbitally with 0.05 cc of sesame oil. Controls, which did not receive atropine, were injected intraorbitally with sesame oil. The controls all died within 2 or 3 minutes after injection. The atropinized mice all lived, one was subsequently injected with 0.05 cc of oil in the other orbital cavity and continued to live. The atropine paralyzes the vagus nerve and thus prevents the oculo-cardiac reflex. It is the blocking of this reflex which enables the mice to survive the intraorbital injections.

Conclusion. It was concluded that the oculo-cardiac reflex was responsible for the sudden and spectacular death of mice injected intraorbitally with minute quantities (0.02 to 0.05 cc) of sesame oil. Mice of cancer susceptible strains succumbed as readily as mice of cancer resistant strains.

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14684

Blood Agglutinins in Blackwater Fever.

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The serums from two cases of blackwater fever were tested for content of α, β, and cold agglutinins.* To 2 drops of the serum dilutions one drop of a 2% suspension of Groups A, B, and O erythrocytes was added. The suspensions in Wassermann tubes were incubated in a water bath at 37°C for 30 minutes followed by storage in the icebox at 6°C overnight. Examinations of suspensions for the presence of agglutinated erythrocytes was done after exposure to 37°C and 6°C with the aid of a dissecting microscope at a magnification of ×20.

Since both serums contained α and β agglutinins, it was necessary to absorb these in order to test for cold agglutinins. Small portions of serums were treated three times with one-half volume of packed fresh Group AB erythrocytes. During each absorption, the mixture was incubated at 37°C for 30 minutes, then centrifuged and the supernatant was treated again with fresh Group AB erythrocytes. Small portions of serum were also treated with a polysaccharide isolated from *Ascaris suum* which has been shown to inhibit specifically the α and β agglutinins in human serums.[1] Enough of the polysaccharide was added to make a 4% suspension. The mixture was incubated at 37°C for 30 minutes and then tested. The serums treated with the Group AB erythrocytes and with the ascarid polysaccharide, as well as the untreated control serums, were then tested with Group A, B, and O erythrocytes and examined after exposures to 37°C for 30 minutes and at 6°C overnight.

Table I shows that the serums from the 2 cases of blackwater fever had markedly increased α isoagglutinin titers of 1:8112 and 1:2028, respectively. After treatment of the serums 3 times with the Group AB erythrocytes, as well as after a single treatment with the ascarid polysaccharide, no α, β, or anti-O agglutinins were detected after incubation at 37°C. However, agglutination was still observed with Groups A, B, and O erythrocytes after overnight exposure to 6°C (Table I).

Discussion and Summary. A polysaccharide has been isolated from various animal parasites which inhibits the α and β agglutinins from human serums and which thus bears some relationship to the human isoagglutinogens.[2] Since the polysaccharide alone is able to incite antibody formation, immunization of the host with this substance during infection might lead to the formation of agglutinins which might act on the erythrocytes of the host. This fact may be of importance in blackwater fever. In this disease, which is associated with infection with *P. falciparum*, there is an intravascular agglutination of erythrocytes followed by extensive intravascular hemolysis. Autoagglutination may be caused by autoagglutinins resulting from the immunization of the host with the substance in the parasite related to the human isoagglutinogens. The increase in the agglutinin titer in the serums from patients suffering with blackwater fever presented in this study and the increase in the α and β agglutinin titers in the serums from patients having had repeated attacks of malaria[2] give evidence to support the view that the malarial organism is responsible for the increase in agglutinins. How these agglutinins resulting from infection may be associated with autoagglutination, however, is not evident from the present results.

The serums from the cases of blackwater fever also had agglutinins acting at 6°C.

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* The author is indebted to Dr. V. Rippert from Caripito Hospital, Creole Petroleum Corporation, Caripito, Venezuela, S.A., for the serums from the cases of blackwater fever.

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[2] Oliver-González, José, and Torregrosa, Vicente M., *J. Infect. Dis.*, 1944, **74**, 173.

TABLE I.
The α, β, and Cold Agglutinin Titers of Serums from Two Cases of Blackwater Fever.

	Titer at 37°C for 30 min Agglutinins			Titer at 6°C overnight Agglutinins		
	α	β	anti-O	α	β	anti-O
Serum sample 1						
Untreated serum	8,112	128	0	32,448	1,024	32
Serum treated with Group AB erythrocytes	0	0	0	32	32	32
Serum treated with ascarid polysaccharide	0	0	0	32	32	32
Serum sample 2						
Untreated serum	2,028	64	0	8,112	256	32
Serum treated with Group AB erythrocytes	0	0	0	64	32	32
Serum treated with ascarid polysaccharide	0	0	0	32	32	32

These agglutinins behaved as true cold agglutinins since they agglutinated all erythrocytes regardless of blood group, and their action was reversible by changing the temperature back and forth from 6°C to 37°C. The presence of autoagglutinins in blackwater fever has been previously suggested although not proved experimentally by Dameshek.[3] As explained previously, the source of the agglutinogen may be the malarial parasite.

The possible relationship between the presence of cold agglutinins and the development of blackwater fever should also be taken into consideration. Since exposure to cold is one of the conditions known to accelerate the appearance of blackwater fever symptoms, it seems that such a relationship could profitably be studied.

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[3] Dameshek, W., *J. A. M. A.*, 1943, **123**, 77.

14685

Acute Toxicity of Choline Hydrochloride Administered Intraperitoneally to Rats.*†

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Choline hydrochloride is not highly toxic. Mott and Halliburton[1] emphasize the low order of toxicity when they state that "We have never succeeded in killing an animal by injection of choline or choline hydrochloride." However, lethal doses for several species have been reported[1-9] as of the order of 40-60 mg per kg body weight for intravenous administration and of the order of 200-1000 mg per kg for subcutaneous administration.

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† This paper was presented in part before the Division of Biological Chemistry at the 106th meeting of the American Chemical Society, Pittsburgh, Pennsylvania, September, 1943.

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[3] Asher, L., and Wood, H. C., *Zeit. Biol.*, 1899, **37**, 307.

TABLE I.
Dosage-Mortality Table for Rats Grouped by Dosage Ranges.*
(Intraperitoneal Injection: 518 Rats.)

	Concentration of choline hydrochloride solutions							
	200 mg/ml		100 mg/ml		40 mg/ml		20 mg/ml	
Dosage range mg/100 g body wt	No. rats	% mortality	No. rats	% mortality	No. rats	% mortality	No. rats	% mortality
20-24	15	0						
25-29	44	25						
30-34	31	61	22	32				
35-39	46	72	43	54	8	12		
40-44	15	100	28	79	43	19		
45-49					39	41		
50-54			20	80	11	82	9	67
55-59			5	60			30	43
60-64					15	87	22	64
65-69							6	100
70-74							14	36
75-79							23	71
80-84					15	100		
85-89							14	86
Range of LD_{50}	29-34 mg/100 g body wt		37-38 mg/100 g body wt		41-49 mg/100 g body wt		59-75 mg/100 g body wt	

* The statistical work was performed by Elizabeth Street, her assistance is gratefully acknowledged.

In the first series of tests, 4 solutions of choline hydrochloride (Eastman Kodak Co.) of concentrations 200, 100, 40 and 20 mg per ml, respectively, were given several groups of rats. These rats were of various ages and body weights. The LD_{50}'s calculated by the methods of Bliss[10] were found to be of the order of 35, 41, 54, and 74 mg per 100 g body weight, respectively for the 4 concentrations given above. These data were criticized on several grounds by Professor J. H. Burn during a visit to this laboratory. Following his suggestions the experiment was repeated in such a way that on any testing day, at least 4 groups of rats of the same body weight were used. For each such 4 groups of rats, 1 group received each of the choline hydrochloride solutions as before. An attempt was made on each test to obtain approximately identical percentage kills from doses of each concentration.

A large series of rats were studied and found to show about the same order of dependence of mortality on concentration as had been obtained in the earlier, less well controlled test. The scatter in the data was so serious that LD_{50} values are given only as ranges (bottom, Table I—combined data from both series). It is clear that the greater the concentration of choline hydrochloride in solution, the less of the drug is required to kill the average rat.

The symptoms of acute choline poisoning have been described in detail elsewhere. A few points bear additional discussion. The lethal effect is extremely rapid following high doses—in many groups of 15 rats receiving such doses all the rats which died had done so within 5 minutes of injection. Changes in respiration in these rats were closely followed by trembling, convulsive movements, salivation, hemorrhage around the eyes in about two-thirds of the rats (Fig. 1) dying from the drug, cyanosis, respiratory paralysis, and death. The dark stain around the eyes was

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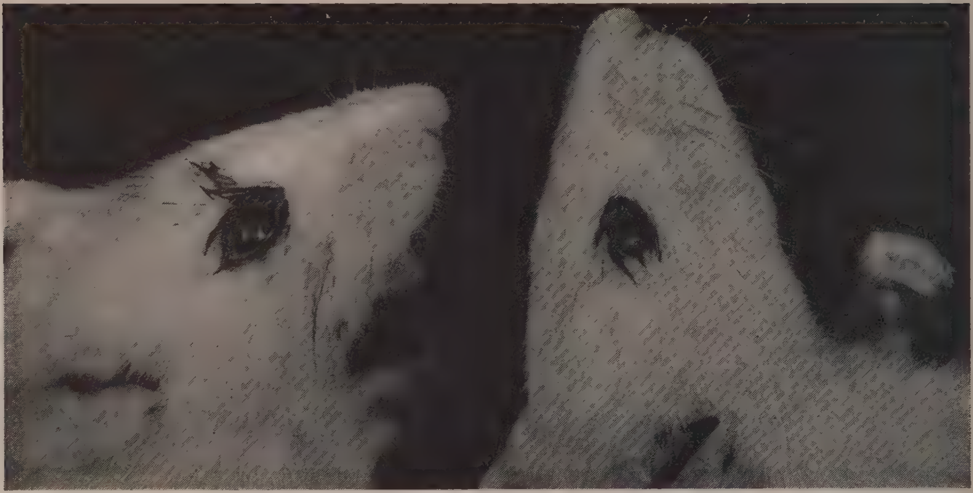

FIG. 1.

Hemorrhage around the eyes after large doses of choline hydrochloride. The bleeding seemed to start from the medial canthus. Excessive salivation may be noted on the lower jaw of rat on the left.

identified as blood by Dr. H. R. Brown, Jr. On first examinations, no red cells were found and we were on the point of concluding, as has Barnard recently,[11] that some porphyrin was responsible. However, more careful and prompt examinations showed the presence of rapidly hemolyzing red blood cells in a saline suspension of the exudate. The saline suspension gave a positive guaiac test. In a stained smear, small red blood cells were found and several polymorphonuclear leucocytes identified. This hemorrhage may be attributed to the intense parasympathomimetic action of overwhelmingly large doses of choline.

Rats which survived 20 minutes after injection of any dose invariably lived. This ability of the organism to excrete or to metabolize and detoxify large amounts of choline has been repeatedly noted. No symptoms of poisoning were evident in surviving rats at any time during an observation period of a week following the various tests.

Summary. 1. The amount of choline hydrochloride to kill the average rat (LD_{50}) is greater the more dilute the solution in which it is injected intraperitoneally.

2. Expressed as mg per 100 g body weight, the LD_{50}'s are as follows: 29-34 for a solution containing 200 mg of choline per ml; 37-38 for a solution of 100 mg per ml; 41-49 for a solution of 40 mg per ml; and 59-75 for a solution of 20 mg per ml.

The author acknowledges the valuable criticisms of Dr. J. H. Burn and wishes to thank Ray Kesel for assistance in handling the animals.

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14686

Chronic Toxicity and Hypercalcemic Effects of Various Activated Sterols in the Albino Rat.

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Toxicity studies on the various activated sterols have generally been carried out on white mice according to the technic of Holtz, Laqueur, Kreitmair, and Moll:[1] a determination of the so-called "toxic borderline dose" or "T.B.D." Data available in this laboratory indicate that the T.B.D.'s of vitamins D_2, D_3, and dihydrotachysterol are, respectively, 50 γ, 80 γ, and 6 γ, based on the weights of the crystalline substances.* The values reported in the literature have been tabulated by McLean,[2] and are of the same general magnitude.

Recently Mrazek, Novak, and Reed[3] reported a study of the chronic toxicity of vitamins D_2 and D_3 for the albino rat. Dosages of 75 I.U. per g per day were given to selected groups of 9 rats until all had died. They found that the average survival time of rats treated with vitamin D_2 was 43 days, and with vitamin D_3, 22 days. Thus it appears that vitamin D_3 is more toxic for the rat than vitamin D_2, but as pointed out above, less toxic than D_2 for the mouse. Morgan, Shimotori, and Hendricks[4] also concluded that vitamin D_3 is more toxic for rats than D_2, but Jung[5] concluded that the toxicities were identical.

The purpose of this study has been to compare, in the albino rat, the toxic and hypercalcemic effects of crystalline vitamins D_2 and D_3 and of commercial dihydrotachysterol with the object of determining whether there is any causal relationship between the two effects. This point cannot be regarded as settled at the present. It is the belief of Reed,[6] for example, that the toxic and hypercalcemic effects of vitamin D are not causally related. The hypercalcemic effects of single doses of the preparations named have been studied by McChesney and Kocher,[7] but data indicating their relative effects on continued administration to the rat are not available.

Experimental Methods. Albino rats of both sexes from our regular breeding colony served as experimental subjects. They ranged in age from 225 to 275 days at the start of the experiments, and weighed from 300 to 600 g. They were given our regular breeding diet[8] (Analysis: Ca, 0.49%; P, 0.53%) and water, *ad libitum*. The medications were given by stomach tube in corn oil, and each rat received 0.125 cc per 100 g body weight per day. Medications were not given on Sundays, therefore 0.1875 cc per 100 g was given on Saturdays and Mondays. (The concentrations of the solutions used ranged from 0.8-2.4 mg/cc.) The rats were weighed twice weekly and the doses were adjusted to these weights. Medications were continued until the animals died. The results of the experiments on chronic toxicity are given in Table I.

In order to determine the hypercalcemic effects of the activated sterols when administered daily, the same procedures were used. The animals were sacrificed by decapitation after 14 days of treatment since it was at

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[1] Holtz, F., Laqueur, F., Kreitmair, H., and Moll, T., *Biochem. Z.*, 1931, **237**, 247.

* The commercial solution of dihydrotachysterol (Hytakerol) contains about 1250 γ of the active principle per cc. Hytakerol is a registered trademark of the Winthrop Chemical Co.

[2] McLean, F. C., *J. A. M. A.*, 1941, **117**, 609.

[3] Mrazek, R., Novak, C. R., and Reed, C. I., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 49.

[4] Morgan, A. F., Shimotori, N., and Hendricks, J. B., *J. Biol. Chem.*, 1940, **134**, 761.

[5] Jung, A., *Schweiz. med. Wochschr.*, 1943, **73**, 17.

[6] Reed, C. I., Struck, H. C., and Steck, I. E., *Vitamin D: Chemistry, Physiology, Pharmacology, Pathology, Experimental and Clinical Investigations*, Univ. of Chicago Press, 1939, p. 152.

[7] McChesney, E. W., and Kocher, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 156.

[8] Climenko, D. R., and McChesney, E. W., *ibid.*, 1942, **51**, 157.

TABLE I.
Survival of Albino Rats Receiving Various Activated Sterols Daily Until Death.

Preparation	Experiment	No. of rats	Dose, mg/kg/day	Survival time, days: Range	Survival time, days: Avg (median)	Equivalent toxic dose[*]
Vitamin D_2	A[§]	12	2.0	13-87	33 ± 5[‡] (22)	3.60
” ”	C	12	2.4	16-72	43 ± 3.3 (42)	
” ”	D	10	3.0	11-82	33 ± 5.6 (25)	
” D_3	A	12	2.0	9-32	18 ± 1.5 (17)	2.30
” ”	B	8	1.5	19-124	59 ± 10 (44)	
” ”	B	8	2.0	16-43	29 ± 2.5 (28)	
” ”	C	11	1.75	12-70	39 ± 4.3 (34)	
” ”	D	10	2.25	7-74	27 ± 4.7 (20)	
Dihydrotachysterol	A	12	1.0	8-181[†]	34 ± 10 (18)	1.00
”	C	12	1.12	9-67	24 ± 2.6 (20)	
”	D	10	1.25	9-35	15 ± 5.4 (12)	

[*] Mg/kg/day—permitting a median 20-day survival time, as estimated from the data of Experiments A, C, and D.

[†] Animal sacrificed on the 181st day and serum Ca found to be 14.3 mg%. Judging from its condition the animal might still have survived for a considerable period of time.

[‡] Probable error of the mean.

[§] The experiment numbers are given in order to indicate which animals were run at the same time.

TABLE II.
Final Serum Calcium Values of Albino Rats Medicated Daily with Various Activated Sterols for 14 Days.

Preparation	No. of rats	Dose, mg/kg/day	Serum calcium, mg%: Range	Serum calcium, mg%: Avg	Dose required to produce serum Ca 15.3 mg%[‡]
Vitamin D_2	8	2.0	13.1-17.3	15.74 ± 0.38[*]	1.85
” D_3	8	1.0	13.5-15.4	14.61 ± 0.23	1.2
” ”	8	1.5	15.3-17.1	16.30 ± 0.16	
Dihydrotachysterol	8	1.0	14.2-17.0	15.29 ± 0.24	1.0

[*] Probable error of the mean.

[†] Estimated from the data, as mg/kg/day for 14 days, assuming that the rise above the normal level of 11.2 mg% is proportional to the log of the dose.[12]

about this time that deaths generally began to occur in the chronic toxicity experiments. Blood was collected from each animal in a separate centrifuge tube, and calcium was determined on the serum by the method of Clark and Collip.[9] The results are given in Table II.

Discussion. The results as to the toxicity of vitamins D_2 and D_3 for the rat are in good agreement with the work of Mrazek, Novak and Reed, considering that slightly higher doses were used in this work. It is further established that dihydrotachysterol, on a weight basis, is more toxic than either vitamin

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[9] Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

[12] McChesney, E. W., and Kocher, H., *Endocrinology*, 1942, **30**, 787.

D_2 or D_3.

The toxicities of the 3 preparations do not completely correlate with their ability to elevate serum calcium. In the case of vitamins D_2 and D_3 the correlation is as satisfactory as can be expected from the experimental procedures: an equivalent toxic dose of vitamin D_2 is 57% greater, and an equivalent hypercalcemic dose 54% greater than that of vitamin D_3. However, if the toxicity of dihydrotachysterol depended on its hypercalcemic effect alone, one would expect the equivalent toxic dose (as determined experimentally) to be about 1.6 mg/kg/day instead of 1 mg/kg/day. This might have been anticipated since dihydrotachysterol as marketed is a mixture and its physiological action may be the summation of the effects of several substances.[10] It is difficult to conceive of crystalline materials such as vitamins D_2 and D_3 containing a hypercalcemic and an antirachitic principle distinct from each other. Correll and Wise,[11] using vitamin D from cod liver oil, were able to produce toxic effects in chicks without raising the blood calcium. On the other hand, toxic doses of irradiated ergosterol and of dihydrotachysterol did elevate the serum calcium.

The survival times reported must be considered as approximate since individual animals or groups of animals are evidently rather variable in their responses from time to time, due to factors beyond control. For example, in 2 experiments with vitamin D_2 the average survival times were 33 and 42 days although in the latter case the daily dose was 20% larger. Attention is also called to the fact that a small decrease in dose level may result in a disproportionate lengthening of the survival time. Thus, in Experiment B a decrease of 25% in the daily dose more than doubled the survival time. If the dose is not sufficiently large to kill the animals within the first three weeks, they appear to develop a tolerance which may permit them to survive for a considerable period of time. For this reason it is felt that the median survival times may be more significant than the averages.

Summary. For the albino rat, medicated daily until death, crystalline vitamin D_3 is about 55%, and commercial dihydrotachysterol about 260% more toxic than crystalline vitamin D_2 (based on the actual weights of the active principles). So far as vitamins D_2 and D_3 are concerned, the toxicities correlate very well with the hypercalcemic effects of the preparations. However, in the case of dihydrotachysterol the toxicity and ability to elevate serum calcium are not in that same proportion. In view of the difference in response of different species, these considerations do not necessarily apply to any species other than the albino rat.

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[10] Werder, F. von, *Z. physiol. Chem.*, 1939, **260**, 119.

[11] Correll, J. T., and Wise, E. C., *J. Nutrition*, 1943, **26**, 641.

14687

Neuromuscular Transmission in Parathyroid Tetany.*

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Calcium is known to be an important factor in the transmission of nerve impulses. It has been shown that in its absence there is a failure of the liberation of acetylcholine at the synapse which otherwise occurs on stimulation of the nerve.[1] In view of this it was considered interesting to study neuromuscular transmission during hypocalcemia produced

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* This work was performed with the aid of a grant from the Rockefeller Foundation.

[1] Harvey, A. M., and MacIntosh, F. C., *J. Physiol.*, 1939-40, **97**, 408.

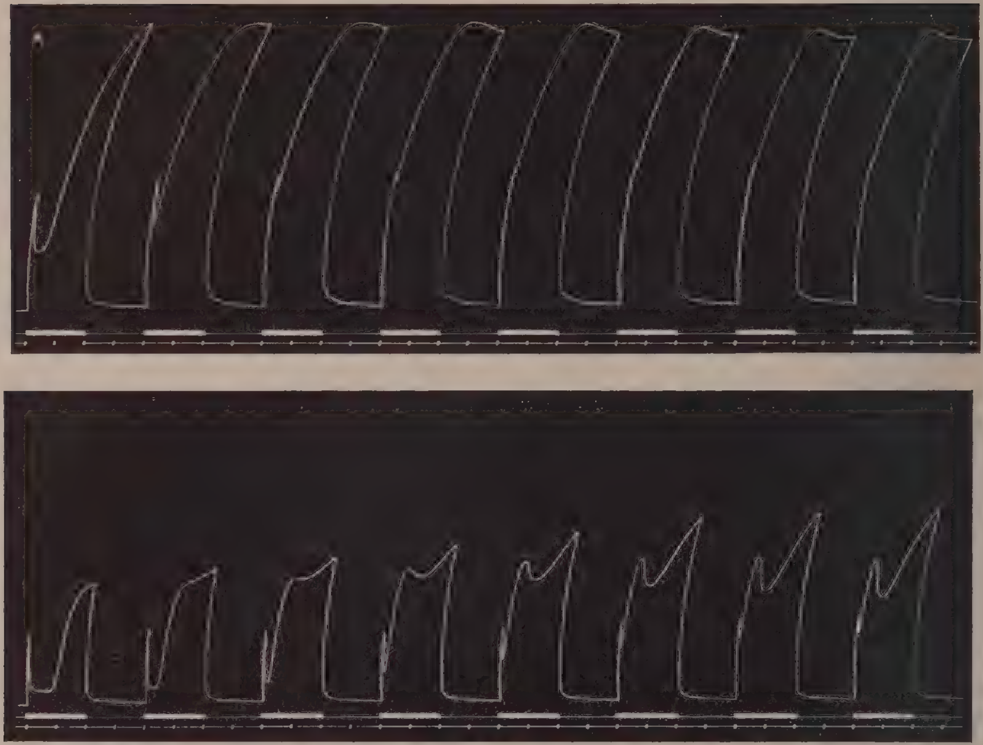

FIG. 1.

Effects of thyroparathyroidectomy in the stages of neuromuscular transmission to repeated stimuli at high frequency, and its normalization after $CaCl_2$ injection.

Cat of 2.5 kg. Anesthesia with nembutal. Thyroparathyroidectomy performed 4 days before. Just before the experiment the animal showed symptoms of tetany. Soleus. Indirect stimulation at 500 per sec. The record is continuous, the stimuli being applied repeatedly for 10 sec., with rest periods of 10 sec.

A. (Top) Before $CaCl_2$ injection. Blood calcium: 6.7 mg%.

B. (Bottom) 30 minutes after 5 cc of 2% $CaCl_2$ injection. Blood calcium: 12.5 mg%.

by removal of the parathyroids.

Method. The thyroids and parathyroids were removed aseptically in 31 cats. Two to 4 days later, when the animals presented clear signs of tetany, they were anesthetized with dial or nembutal. The bones of the legs were fixed by drills, records were taken from the soleus muscle, and the nerve was stimulated with condenser discharges whose frequency was controlled electronically. In 16 animals the blood calcium was determined by the classical method of Kramer and Tisdall. The level of blood calcium shifted between 4.6 mg % and 7.6 mg % (the value of normal cats was about 12 mg %). The effects of thyroidectomy were prevented by giving, in 6 out of the 31 animals, 0.10 g of thyroid extract daily, beginning on the day of operation. In 8 out of the 31 animals, after being tested the neuromuscular transmission and the blood calcium value .2 cc per kilo of a 2% solution of $CaCl_2$ was injected intravenously. And a new test of the neuromuscular transmission was done. The blood calcium 30 minutes after the injection was found to a level between 9.4 mg % and 12.6 mg %.

Results. Indirect stimulation of the muscle produces contractions whose characteristics vary according to the frequency of stimulation. We have fully confirmed the stages of neuromuscular transmission which have been described,[2,3,4] namely, stages 1, 2, 3a, 3b, 3c, 4, and 5. These stages change during parathyroid tetany. Not all of them were uniformly altered in 31 animals studied. Rosenblueth and Cannon[4] showed that repeated periods of 10 to 15 sec. indirect stimulation of the gastrocnemius-soleus at a frequency of 500 per sec., with pauses of 10 to 15 sec., made increasingly clear the series of stages, 1, 2, 3a, and 3b, while the tension developed increased. This phenomenon, confirmed many times in this department, is regularly changed in hypocalcemic animals. Stages 3b and 3c do not appear; the stages do not become more distinct, and the tension does not increase. On the contrary, the muscle shows a marked tendency to fatigue. This always occurred, even when the first period of stimulation produced a response with stages 1, 2, 3a, and 3b.

In order of importance, the other changes are as follows: loss of the characteristic responses at frequencies of stimulation of 500, 400, 300, 200, 100, and 60 per sec. Frequently stage 3c as normally seen with 300 or 150 per sec. does not appear. Marked neuromuscular fatigue often occurs even at a frequency of 60 per sec.

In the group of animals in which the blood calcium was increased to normal values by injecting $CaCl_2$ the synaptic behavior resembles very much that of the normal cats.

Comment. These changes cannot be attributed to the removal of the thyroid glands, since the operated animal which received thyroid extract showed the same changes.

The abnormal synaptic reaction of the parathyroidectomized animals is probably due to the low value of blood calcium during tetany as it is normalized by increasing the blood calcium to normal values.

It is accepted that the chemical transmitter of the neuromuscular synapse is acetylcholine. Harvey and MacIntosh[1] showed that the calcium ion is fundamental for cholinergic transmission, since without this ion, acetylcholine is not released.

The marked fatigability of the neuromuscular synapse in animals with parathyroid tetany may be explained by the work of these authors. The decrease of calcium ion would hinder the release of acetylcholine and thus lead to the failure of transmission.

Conclusions and Summary. The responses of the soleus muscle of the cat on stimulation of its nerve at varying frequencies were studied in animals rendered hypocalcemic by parathyroidectomy. The responses at certain frequencies were markedly altered. These alterations were normalized by calcium administration.

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[2] Rosenblueth, A., and Morison, R. S., *Am. J. Physiol.*, 1937, **119**, 236.

[3] Rosenblueth, A., and Luco, J. B., *Am. J. Physiol.*, 1939, **126**, 39.

[4] Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1940, **130**, 205.

14688

Strain Differences in the Choline Requirement of Rats.*

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In earlier work on choline in this laboratory, 2 strains of rats were used. Rats from these 2 strains appeared to differ in their requirement for choline. In order to determine whether these differences were genetic in character, females from the 2 strains were selected and bred to their respective littermates. The progeny were designated as the F_2 generation. It was previously reported[1] that the F_2 generation of the 2 strains showed significant dif-

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station.

[1] Engel, R. W., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 281.

TABLE I.
Number, Sex, and Kidney Condition of Rats in the F_3 Generation Receiving Various Levels of Choline.

		LCR strain rats				HCR strain rats			
Mg choline per day		2	3	4	6	4	6	8	10
Total number of rats	♂	13	14	19	7	14	15	10	4
	♀	10	18	33	9	15	19	11	4
No. of deaths	♂	4	2	2	0	12	10	0	1
	♀	0	0	0	0	7	7	0	0
No. of survivors showing kidney hemorrhage	♂	4	2	1	0	0	2	2	2
	♀	2	2	0	0	0	1	1	0
No. of survivors showing swollen or frosted kidneys	♂	1	3	2	0	2	3	3	0
	♀	1	2	2	0	7	8	4	0
No. of survivors showing normal kidneys	♂	4	7	14	7	0	0	5	1
	♀	7	14	31	9	1	3	6	4

ferences in their choline requirements. The rats having the higher requirement, designated as strain HCR, were hooded rats selected from the Wisconsin strain; those having the lower choline requirement, designated as strain LCR, were albino rats selected from the Wistar strain. It was desirable to determine whether the observed differences would be transmitted to the F_3 generation, and to measure these differences quantitatively. Brother-sister matings of the F_2 generations in the 2 strains were made, and the progeny were used for quantitative measurements of their respective choline requirements.

Choline Requirement of the F_3 Generation. At the age of 23 days, rats of the F_3 generation of both strains were placed on experimental diet 31PMC,[†] which was supplemented with adequate amounts of thiamine, pyridoxine, riboflavin, calcium pantothenate, inositol, carotene, and calciferol. They received various dosages of choline in order to see if the minimum choline requirement could be established for each strain. HCR strain rats of the F_3 generation were fed 4, 6, 8, and 10 mg of choline daily, and LCR strain animals of the same generation were fed 2, 3, 4, and 6 mg of choline daily. The rats remained on the experimental diet for 14 days. At the end of this period, the surviving animals were killed and their kidneys were examined. Ninety-two rats of the strain HCR and 123 rats of the strain LCR were tested over a period of 6 months.

The results of the experiments are given in Table I. Nineteen of the 29 HCR strain rats on a daily level of 4 mg of choline had fatal kidney hemorrhage; 9 of the survivors had injured kidneys at necropsy and only 1 was normal. Of the 52 LCR rats on the 4 mg daily level of choline, only 2 died and of the survivors 45 were normal. All the LCR strain animals tested on a 6 mg daily level of choline lived and had normal kidneys at necropsy, but this level was far too low for the HCR strain animals. Seventeen of the 34 HCR strain animals placed on the daily level of 6 mg of choline had fatal kidney hemorrhage; 3 had kidney hemorrhage but survived, and only 3 were normal. Even on the extremely low level of 2 mg of choline a day, the incidence of fatal kidney hemorrhage in rats of the LCR strain was not nearly as great as that of HCR strain rats on 6 mg of choline a day. Moreover, only 50% of the HCR animals on a level of 8 mg of choline a day were normal.

It is evident that the choline requirement of the F_3 generation of the strain HCR was more than twice as great as that of the LCR strain animals. On diet 31PMC, the minimum daily choline requirement was approximately 4 to 6 mg for F_3 generation LCR strain rats, and above 10 mg for HCR strain rats of the same generation. In both strains the incidence of kidney hemorrhage was less in females than in males on the same diet and level of choline. All female rats of the F_3 generation of the LCR strain lived for the duration of the experiment regardless of the level of choline on which they were placed. This confirms the results of Griffith[2] and of Engel and Salmon,[3] in which it was reported that choline deficiencies appeared more slowly and were less severe in female than in male rats on the same diet.

[†] The breeding rats of the F_1 and F_2 generations were fed stock diet 30. This diet consisted of ground wheat 64, meat scraps 10, casein 10, alfalfa leaf meal 2, bone meal 2, iodized salt 1, molasses 5, lard 5, and cod liver oil 1%. It contained 6.7 mg of choline per 10 g by analysis. Diet 31PMC consisted of alcohol extracted peanut meal 30, alcohol extracted casein 6, sucrose 54, lard 6, and salt mixture No. 186 4%.

The results of the experiment show the necessity for additions of choline to purified diets for experimental purposes. The fact that the rats may vary in their requirement for specific nutriments, such as choline, is of significance in relation to experimental procedures. The possibility of such variations should also be considered in relation to the practical nutrition of humans and the domestic animals.

Summary. Two strains of rats were selected which showed significant differences in their requirement of choline. Tests on the progeny of each strain proved that this difference continued through the F_3 generation. The minimum daily choline requirement of the strain LCR was approximately 4 to 6 mg per rat on diet 31PMC, and the minimum daily choline requirement of the strain HCR on the same diet was above 10 mg per rat.

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[2] Griffith, W. H., *J. Nutrition*, 1940, **19**, 437.

[3] Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

14689

The Mechanism of Action of Thiouracil.

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According to the investigations of MacKenzie[1] the thyroid-inhibiting action of thiourea, sulfaguanidine, etc., results from direct action on the gland itself whereby the secretion is inhibited and the gland hypertrophies as the restraining influence of the thyroid hormone on the pituitary is eliminated; there is also lowered metabolic rate, interference with growth and development, in other words, all symptoms of hypothyroidism.

These writers also discuss the problem of whether the circulating hormone (thyroxin) may become inactivated by the drug. But they denied this possibility since in thyroidectomized rats thiourea—which does further depress the b.m.r.—is not antagonized by *injected* thyroxin. In other words: thyroxin raises the metabolism of thyroidectomized rats even if thiourea had been administered. Similarly, Astwood, Sullivan, Bissell, and Tyslowitz[2] concluded that artificially administered thyroid hormone is equally calorigenic and toxic in the drug-treated animals as in the controls.

In contrast to these findings, we have found that thiourea (and thiouracil) *do* interfere with the action of *orally administered thyroid*, if the well known Reid Hunt[3] method is made the basis of observations. In this test as little as 1 mg of whole thyroid gland can be detected since it imparts to the mice a distinct resistance against otherwise fatal doses of acetonitrile. This increased resistance is partially removed by thiourea or thiouracil as the following experiments show.

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[1] MacKenzie, C. G., and MacKenzie, Julia B., *Endocrinology*, 1943, **32**, 185.

[2] Astwood, E. B., Sullivan, J., Bissell, Adel, and Tyslowitz, R., *Endocrinology*, 1943, **32**, 210.

[3] Hunt, Reid, *Arch. Int. Med.*, 1925, **35**, 671.

Experimental Methods. White female mice were obtained from our usual commercial source and kept in cages in lots of 3 to a cage. The mice were fed a diet of Pratt's Rabbit Pellets which were ground to a fine powder in a drug mill, and moistened with water so as to form a cake. Each 100 g of dry food was fortified by the addition of 2 cc of cod liver oil USP. After an interval of one week on this diet of 5 g of food per day per mouse, the food, with the exception of the controls, had added to it varying amounts of thyroid, thiouracil and thiourea. Each set of mice was kept under identical conditions for a period of 2 weeks while receiving the diet. At the end of 2 weeks the mice were weighed and given intraperitoneal injection of acetonitrile, dilution 20 mg per cc. Variation of the toxicity of acetonitrile is to be expected according to the season of the year (Munch[4]). Death within 24 hours was taken as the end point.

On our first series of mice (12 to a set) the following results were obtained. All data were compiled following a Karber condensation curve as modified by Muench.[5] LD_{50} is in terms of acetonitrile.

TABLE I.

Diet		LD_{50} mg/kg	% in terms of controls
Controls		500	100
Thyroid	1 mg	800	160
’’	2 ’’	900	180
Thiouracil	2 ’’	500	100
Thiourea	2 ’’	525	105

As is shown by the Table 1 the feeding of thiouracil and thiourea did not decrease the resistance of white mice to acetonitrile.

A second series of mice was run under like conditions. The diet had added to it 1 mg of thyroid and varying amounts of thiouracil and thiourea as indicated in Table II.

TABLE II.

Diet	LD_{50} mg/kg	%
Controls	450	100
Thyroid 1 mg	850	191
’’ 1 ’’ Thiouracil 1 mg	650	144
’’ 1 ’’ ’’ 2 ’’	550	122
’’ 1 ’’ Thiourea 2 ’’	600	133

Results. On the basis of the data obtained in Table I thiourea and thiouracil have no effect in relation to the toxicity of acetonitrile in mice.

On the data obtained in Table II both thiouracil and thiourea reduce the LD_{50} per kilo of mouse. In terms of percentage, 1 mg of thyroid protects 91% more than the control mice. When 1 mg of thiouracil is added to the daily diet the protection ratio dropped 47%. When 2 mg of thiouracil was added daily the protection ratio dropped 69%. Thiourea 2 mg reduces the protection ratio 58%. On the basis of these results it would appear that about 3 to 4 mg of thiouracil will neutralize the effect of 1 mg of whole thyroid gland on white mice. Thiourea is not quite as efficient as thiouracil.

Conclusion. Thiouracil and thiourea in addition to preventing the synthesis of the thyroid hormone has some effect on thyroid *per se* when fed to white mice.

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[4] Munch, J. C., *Bioassays,* 1931, 672,

[5] Reed, L. J., Muench, H., *Am. J. Hygiene,* 1938, **27**, 493.

14690

Inhibitory Action of Thiouracil, Thiocarbamide and Other Compounds on Melanin Formation by Tyrosinase.

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Sulfonamides, thiocarbamide, and a number of other compounds containing —NH·CS·NH— or $NH_2 \cdot C_6H_4$— groups have been found to suppress formation of thyroid hormone.[1,2] Because of indications that this action depends upon interference with an oxidative enzymatic process,[3] it was thought to be of interest to study the action of these and similar agents on enzyme systems *in vitro*. The present report deals with the influence of thiouracil† and thiocarbamide on the tyrosinase-tyrosine system as compared to that of glutathione, cysteine, sulfonamides, *p*-aminobenzoic acid and ascorbic acid.

Methods. The technic of Baker *et al.*[4] was employed, the substrate being a 0.001 M solution of tyrosine in phosphate buffer pH 7.0. The agents studied were dissolved in this substrate in 0.001 M concentration. A crude tyrosinase solution was prepared by grinding potatoes and centrifuging the mash. The volumes of the solutions employed are indicated in the tables. The solutions were observed for 2 hours, then hourly for 7 hours, and again after 24 hours.

Results and Comment. The pertinent data are presented in the tables. Inhibition of melanin formation by *p*-aminobenzoic acid and sulfanilamide has been reported by Martin *et al.*[5] A similar effect is produced by sodium sulfathiazole and sulfadiazine. The inhibiting action of glutathione has been described by Figge[6] and that of phenylthiocarbamide and thiocarbamide by Bernheim and Bernheim[7]. We found thiouracil to be the most potent in this regard of the compounds investigated by us. It is also a more potent anti-thyroid agent than thiocarbamide, *p*-aminobenzoic acid and sulfonamides.[8] Ascorbic acid exerts no anti-thyroid effect;[8] the influence of glutathione and cysteine in this connection has not been studied. The absence of strict parallelism between *in vitro* tyrosinase-inhibiting effect and *in vivo* thyroid-inhibiting effect is indicated by the fact that phenylthiourea is 10-20 times as potent as thiourea in inhibiting tyrosinase[7] whereas phenylthiourea has no effect and thiourea a strong anti-thyroid effect in the rat.[8]

The influence of glutathione and cysteine is of interest in view of the demonstration by György[9] that sulfhydryl compounds as well as thiocarbamide and thiouracil inhibit autooxidation (rancidity) of fat. He offers the hypothesis that this antioxygenic action of thiocarbamide is due to the sulfhydryl radicle in its tautomeric form (isothiocarbamide), and considers the possibility that the action of these agents as antioxidants may explain their anti-thyroid activity. Our findings do not support this hypothesis when applied to inhibition of tyrosinase activity. As indicated in Table I, melanin formation was completely inhibited by thiouracil in 0.0005 M concentration and only partially inhibited by

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* J. Ewing Mears Fellow in Physiology and Medicine.

[1] Mackenzie, C. G., and Mackenzie, J. B., *Endocrinol.*, 1943, **32**, 185.

[2] Astwood, E. B., Sullivan, J., Bissell, A. B., and Tyslowitz, R., *Endocrinol.*, 1943, **32**, 210.

[3] Dempsey, E. W., *Endocrinol.*, 1944, **34**, 27.

† Thiouracil used in this study was supplied by the Lederle Laboratories through the courtesy of Dr. B. Carey and Dr. S. M. Hardy.

[4] Baker, A. K., Kline, B. E., and Rush, H. P., Proc. Soc. Exp. Biol. and Med., 1942, **50**, 361.

[5] Martin, G. J., Wisansky, W. A., and Ansbacher, S., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 26.

[6] Figge, F. H. J., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 269.

[7] Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, 1942, **145**, 213.

[8] Astwood, E. B., *J. Pharm. Exp. Therap.*, 1943, **78**, 79.

[9] György, P., Stiller, E. T., and Williamson, M. B., *Science*, 1943, **98**, 518.

TABLE I.
Relative Potency of Various Inhibitors.
Melanin Formation. 7 Hours.

	0.001 M	0.00075 M	0.0005 M	0.00025 M
Thiouracil	0	0	0	+
Thiocarbamide	±	++	+++	+++
p-Aminobenzoic acid	0	+	++	+++
Sod. Sulfathiazol	0	+	++	++
Sulfadiazine	0	+	++	++
Glutathione	0		++	

Substrate: 20 cc 0.001 M tyrosine solution in phosphate buffer pH 7.0. Inhibitors dissolved in tyrosine solution. Enzyme solution: 0.5 cc.

TABLE II.
Inhibition of Melanin Formation in 0.001 M Tyrosine Solution.

						Melanin	
	Inhibitors	Volume, cc	Molarity, M	Activator	Pink (Hallachrome?)	7	24 hr
1.	0			0	10 min	+++	++++
2.	Thiouracil			0	0	0	0
	2.5 mg per	20	0.001	Iodoacetic acid	trace 60 min	0	0
	0.5 cc enzyme			$CuSO_4$		0	0
3.	1.2 mg per	10	0.001	0	" 30 "	0	0
	0.5 cc enzyme			Iodoacetic acid	" 30 "	+	+++
				$CuSO_4$		++	++++
4.	Thiocarbamide			0	10 min	+	+
	1.6 mg per	20	0.001	Iodoacetic acid	10 "	+	+
	0.5 cc enzyme			$CuSO_4$		++	+++
5.	0.8 mg per	20	0.001	0	10 "	+	+
	0.5 cc enzyme			Iodoacetic acid	10 "	+	+++
				$CuSO_4$		+	++++
6.	Glutathione			0	0	0	0
	6.1 mg per	20	0.001	Iodoacetic acid		0	+
	0.5 cc enzyme			$CuSO_4$		++	++
7.	3.0 mg per	10	0.001	0		0	++++
	0.5 cc enzyme			Iodoacetic acid		+	++++
				$CuSO_4$		+	++++
8.	1.5 mg per	10	0.0005	0		++	++++
	0.5 cc enzyme			Iodoacetic acid		++	++++
				$CuSO_4$		++	++++
9.	Cysteine HCl			0	30 "	++	+++
	1.2 mg per	10	0.001	Iodoacetic acid	30 "	+++	++++
	0.5 cc enzyme			$CuSO_4$		++	++++
10.	Ascorbic acid			0	0	0	0
	3.5 mg per	20	0.001	Iodoacetic acid	0	0	0
	0.5 cc enzyme			$CuSO_4$	0	0	0

glutathione in the same concentration. If it be assumed that 50% of the thiouracil is present in its tautomeric form, the glutathione solution is a weaker inhibitor than the thiouracil solution containing one-fourth the number of —SH groups.

Ascorbic acid was included in this study because of the contradictory results obtained by other investigators. Evans and Raper[10] found that ascorbic acid inhibited melanin formation, whereas Figge[11] observed increased pigment formation in the presence of this agent. We have found that ascorbic acid in 0.001 M solution (column 10, Table II), inhibits melanin formation as completely as thiouracil in equimolecular concentration (column 2, Table II).

In an attempt to gain more information regarding the mechanism of inhibition of melanin production by these substances, the possible anti-inhibiting influence of certain agents was investigated. Tyrosinase is known to be a copper proteinate[12] and it is conceivable that the inhibitors may combine with the prosthetic copper group. Addition of $CuSO_4$ to a tyrosinase-tyrosine solution neither hastened nor intensified melanin formation, but the inhibitory effect of glutathione, thiocarbamide, and thiouracil was to some extent overcome by copper. Data presented in Table II indicate that at higher inhibitor/enzyme ratios the inhibitory effect of glutathione and thiocarbamide was still overcome somewhat by copper, but that of thiouracil and ascorbic acid was not affected, demonstrating the higher inhibiting potency of the latter compounds.

In view of György's suggestion[9] that the antioxygenic action of thiocarbamide may be due to its tautomeric form isothiocarbamide, the effect of iodoacetic acid which inactivates —SH, was investigated. The results obtained were identical with those obtained with copper (Table II).

Raper[13] has shown that tyrosinase is necessary only for the initial phases of melanin formation, *i. e.,* for oxidation of tyrosine to dopa and hallachrome, the subsequent stages proceeding in the absence of the enzyme. We have observed a trace of pink color, probably due to hallachrome, in some of the experiments in which inhibition of melanin formation was complete. It was therefore thought possible that this inhibition might be due to interference with the later, non-enzymatic phases of melanin formation rather than to an anti-enzyme action. However, this seems unlikely, because the melanin precursor derived from hallachrome is tan-colored[11] and should have been detectable in our observations. A tan color developed with the sulfonamides and *p*-aminobenzoic acid, but not with thiouracil, thiocarbamide, glutathione or ascorbic acid. The possibility that the reaction might be arrested at the stage of a colorless compound cannot be excluded.

Summary and Conclusions. 1. Melanin formation in a tyrosinase-tyrosine system was inhibited by thiouracil, thiocarbamide, glutathione, cysteine, ascorbic acid, *p*-aminobenzoic acid, and sodium sulfathiazole and sulfadiazine. 2. This inhibition is overcome by copper and by iodoacetic acid. 3. Thiouracil and ascorbic acid were more potent inhibitors than the other compounds studied. 4. Available data do not permit a definite statement regarding the nature of the inhibitory mechanism, but they suggest a direct anti-enzyme action.

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[10] Evans, W. C., and Raper, H. S., *Biochem. J.*, 1937, **31**, 2162.

[11] Figge, F. H. J., *J. Cell. Comp. Physiol.*, 1940, **15**, 233.

[12] Kubowitz, F., *Biochem. Z.*, 1938, **296**, 443.

[13] Raper, H. S., *Erg. Enzymforsch.*, 1932, **1**, 720.

14691

Nature and Role of the Lytic Factor in Hemolytic Streptococcal Fibrinolysis.*

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The dissolution of human plasma-clot by cultures or culture-filtrates of Group A β-hemolytic streptococci was observed by Tillett and Garner.[1] The active agent, termed fibrinolysin, was also capable of lysing preparations of fibrinogen, as was indicated by the loss of clotting ability of the fibrinogen and by the alteration of its properties of solubility, heat coagulability,[2] and antigenicity.[3] It was not active on casein, gelatin, or peptone, and only slightly active on fibrins of animal origin.[4]

The action of fibrinolysin on human fibrin was accompanied by a slight increase in amino nitrogen. Since the end-product remained protein in nature, Garner and Tillett[2] concluded that the degradation of the fibrin molecule was slight. They stated that fibrinolysin was probably an enzyme, but that its action was specific for fibrinogen and fibrin of human origin.

Milstone[5] demonstrated that human fibrinogen, purified by repeated precipitation, was not affected by fibrinolysin, but that the process of streptococcal fibrinolysis required the presence of an accessory *lytic factor* associated with the euglobulin component of human serum. Lytic factor preparations *per se* generally showed no lytic activity on fibrinogen or fibrin.

In recent blood coagulation studies, Tagnon, Davidson, and Taylor[6] isolated from normal human plasma a euglobulin component, *globulin substance,* which, after treatment with chloroform, possessed both fibrinogenolytic and fibrinolytic properties. Subsequent studies[7] demonstrated that the chloroform-activated *globulin substance* was a true tryptic enzyme capable of digesting gelatin, casein, and hemoglobin.

Since the two substances, lytic factor and globulin substance, were associated with the euglobulin component of the serum proteins, since each was obtained from normal serum by a similar procedure of dilution and acidification, and since each was apparently a major participant in the fibrinolytic process, it seemed possible that the two preparations contained the same lytic agent. Moreover, though the relation of streptococcal fibrinolysin to the lytic factor remained obscure, it was similar in some respects to the interaction of chloroform and globulin substance.

The purpose of this work, therefore, is to report: (1) a study of the relationship of the lytic factor of Milstone to the serum tryptase, and (2) an investigation of the interaction of fibrinolysin and the lytic factor in an attempt to elucidate further the mechanism of hemolytic streptococcal fibrinolysis.

Materials and Methods. Fibrinolysin Prep-

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* This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation, and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

[1] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

[2] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 255.

[3] Jablonowitz, J. M., *J. Immunol.*, 1939, **36**, 261.

[4] Van Deventer, J. K., and Reich, T., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 821.

[5] Milstone, H., *J. Immunol.*, 1941, **42**, 109.

[6] Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525.

[7] Kaplan, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

aration. The source of the fibrinolysin was a highly active fibrinolytic strain of Group A hemolytic streptococcus isolated from the blood stream of a patient with erysipelas. Fibrinolysin was obtained by the alcohol-precipitation method of Garner and Tillett[8] from a Seitz filtrate of an 18-hour culture in beef-heart infusion broth containing 0.05% added dextrose. The alcohol precipitate was redissolved in 1/100 M phosphate-buffered physiological saline, pH 7.4, and dried in 5 ml lots by the lyophile process. The dried product was highly stable, and upon reconstitution with distilled water, dissolved rapidly and completely.

Fibrinogen. Stable lyophilized preparations of human fibrinogen were obtained through the courtesy of Drs. E. J. Cohn and S. Howard Armstrong, Jr., of the Department of Physical Chemistry, Harvard Medical School.[†] The fibrinogen solution employed contained 0.15% fibrinogen in 1/100 M phosphate buffered saline, pH 7.4. Clots formed from the fibrinogen preparation were readily lysed by fibrinolysin, indicating that the preparation contained lytic factor.

Thrombin. A stable and highly active rabbit thrombin preparation ("Hemostatic Globulin," Lederle Laboratories, Inc.), was employed in a 1:10 dilution.

Lytic factor was prepared by the method of Milstone.[5]

Globulin substance was prepared by the acid precipitation method of Pohle and Taylor,[9] except that either defibrinated plasma or serum was used instead of citrated plasma.

For the determination of proteolytic activity the substrates used were fibrin, hemoglobin, and gelatin.

In the case of fibrin, lysis rate was determined on a standard fibrinogen-thrombin clot formed by the addition of 0.2 ml of a 1:10 dilution of rabbit thrombin to a mixture of 1.0 ml of the standard 0.15% fibrinogen solution and 1.0 ml of the test material. Lysis time was taken as the interval between the clotting time and complete dissolution of the fibrin clot. The clotting time was approximately 3 minutes.

Gelatin liquefaction was measured viscosimetrically by Ostwald viscosity tubes in a constant temperature bath at 37.5°C. The gelatin was a 3% solution in 1/20 M phosphate buffer, pH 7.4; it contained thymol as a bacteriostatic agent. Proteolytic activity was determined as follows: 4.0 ml of gelatin were mixed with 2.0 ml of the test preparation, and 5.0 ml of this mixture were then transferred to a viscosity tube. The temperature of the reagents was adjusted to 37.5°C before mixing. Viscosity efflux times at 37.5°C were determined at 20-minute intervals. Rate of proteolysis was measured by the percent decrease in efflux time of the substrate-enzyme mixture. The initial efflux time was determined by extrapolation to 0 time.

The hydrolysis of hemoglobin was measured by the liberation of tyrosine, as determined colorimetrically by the method of Anson.[10] Merthiolate to a final concentration of 1:10,000 was used as the bacteriostatic agent in the hemoglobin substrate preparations.

Bacteriological cultures on all enzyme-substrate mixtures were negative in both plate and broth culture.

All determinations of pH were made by the glass electrode.

Results. A. *Relation of Lytic Factor to Serum Tryptase.* Globulin substance treated with chloroform possesses proteolytic properties. When lytic factor preparation was incubated with 1/10 its volume of chloroform for 48 hours at 25°C, and the chloroform then removed by centrifugation and aeration, a proteolytic material was elaborated as was indicated by its action on gelatin, hemoglobin, and fibrin (Table I). Globulin substance and lytic factor preparation not treated with

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[8] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

[†] The products of plasma fractionation employed in this work were prepared by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, from blood collected by the American Red Cross, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[9] Pohle, F. J., and Taylor, F. H. L., *J. Clin. Invest.*, 1937, **16**, 741.

[10] Anson, M. L., *J. Gen. Physiol.*, 1938, **22**, 79.

TABLE I.

Proteolytic Activity of Lytic Factor and Globulin Substance Preparations Activated by Chloroform, Alcohol, and Acetone.

Exp. No.	Preparation	Incubated 2 days with 1/10 vol.	Gelatin Change in relative viscosity in 2 hr %	Hemoglobin Increase in tyrosine (equivalents/ml) in 24 hr	Fibrin Lysis time
1	Lytic Factor	Chloroform	12.7	36.6	17 min
2	Globulin Substance	"	5.4	27.6	26 "
3	Lytic Factor	—	0	0	>24 hr
4	Globulin Substance	—	1.1	0	>24 "
5	Lytic Factor	Alcohol	6.3	11.1	80 min
6	Globulin Substance	"	5.2	15.0	10 "
7	Lytic Factor	Acetone	4.1	8.7	90 "
8	Globulin Substance	"	9.1	16.2	10 "

TABLE II.

Proteolytic Activity of the Lytic Factor Activated by Fibrinolysin.

Exp. No.	Preparation	Fibrinolysin, ml	Saline, ml	Gelatin Change in relative viscosity in 2 hr %	Hemoglobin Increase in tyrosine (equivalents/ml) in 24 hr
1	1.0 ml Lytic Factor	0	1.0	1.1	0
2	0 " " "	1.0	1.0	0	0
3	1.0 " " "	1.0	0	23.9	16.8
4	1.0 Globulin Substance	0	1.0	1.0	0
5	1.0 " "	1.0	0	26.6	13.2

chloroform demonstrated little or no activity (Exp. 3 and 4, Table I).

When the two preparations were made from equal volumes of defibrinated plasma, the amount of activity on hemoglobin in both cases was fairly comparable at the end of 24 hours, as indicated by the tyrosine levels at this point.

Liberation of the serum tryptase by other organic solvents such as alcohol and acetone has been reported.[11] The incubation of lytic factor and globulin substance with 1/10 volumes of alcohol and acetone also resulted in the elicitation of proteolytic activity. (Exps. 5, 6, 7, 8, Table I).

The lytic factor preparation of Milstone may then be activated by chloroform and other organic solvents to yield a true proteolytic enzyme. The liberated tryptase is comparable in its activity on the substrates studied to the enzyme obtained from globulin substance. Both lytic factor and globulin substance preparations are comparable in their quantitative content of the proteinase.

Lytic factor was originally obtained by Milstone from diluted serum at an acid reaction corresponding at pH 5.0-5.2; globulin substance was obtained by Pohle and Taylor from diluted plasma at pH 6.0.

The results obtained above have indicated that the iso-electric precipitates at the two close pH values probably contain the same lytic constituent.

B. *Fibrinolysin-Lytic Factor Reaction.* Milstone reported that the addition of lytic factor preparation to purified fibrin was without effect, but on the admixture of fibrinolysin, clot dissolution occurred readily. To determine whether this was a case of true proteolytic action, each of the following—fibrinolysin, lytic factor preparation, and a lytic factor-fibrinolysin mixture—was incubated with gelatin, hemoglobin, and fibrin. Lytic factor alone and fibrinolysin alone were without effect; but in the presence of both sub-

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[11] Stephan, R., and Wohl, E., *Z. exp. Med.*, 1921, **24**, 391.

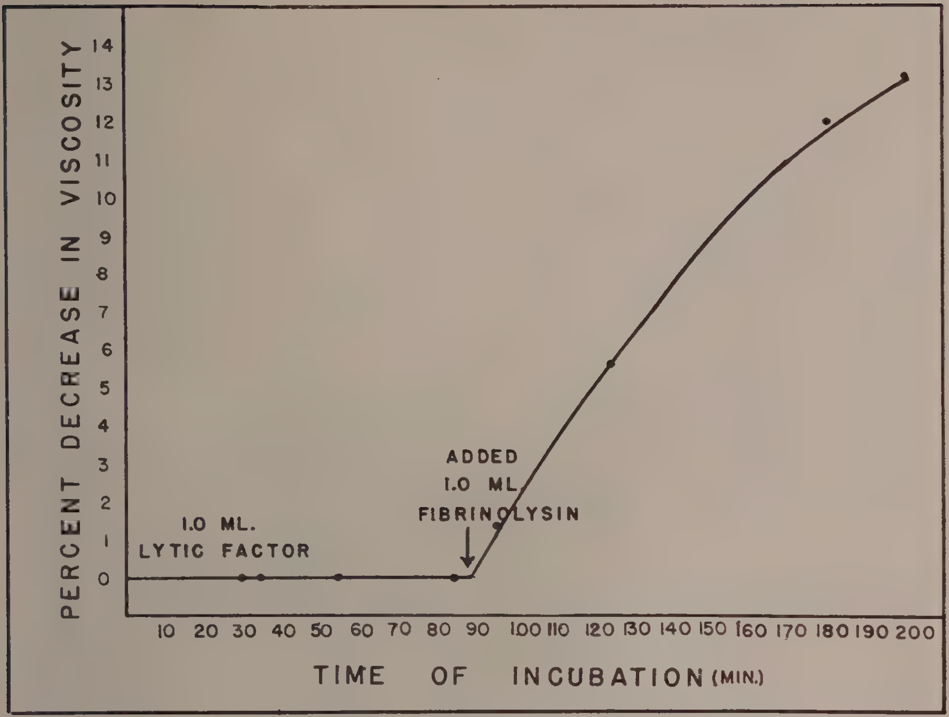

Fig. 1.
Activation of the lytic factor by fibrinolysin with gelatin as substrate.

stances, hydrolysis of gelatin and hemoglobin was marked (Table II) (Fig. 1).

Identical results were obtained when globulin substance was used instead of lytic factor (Exps. 4, 5, Table II).

In no case was fibrinolysin alone active on gelatin or hemoglobin substrates; proteolysis occurred only upon the concomitant action of fibrinolysin and lytic factor. However, since lytic factor itself could be converted by chloroform, alcohol, or acetone to an active proteinase, it must be concluded that the function of fibrinolysin then involves the activation of the serum tryptase from an inert precursor. Christensen,[12] in a recent abstract, has independently reported activation of the lytic factor by fibrinolysin.

The chemical basis for the activation reaction is obscure. In general, activation by the organic solvents proceeds at an exceedingly slow rate, while activation by fibrinolysin is comparatively rapid and suggestive of a monomolecular catalytic reaction. Studies of the chemical nature and the kinetics of the lytic factor-fibrinolysin reaction are being continued.

Summary. The lytic factor of Milstone and the globulin substance may both be activated by certain organic solvents to yield a true proteolytic enzyme. Both lytic factor and globulin substance are comparable in their quantitative content of the proteinase.

Fibrinolysin was found to activate the serum tryptase from an inert precursor.

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[12] Christensen, L. R., *J. Bact.*, 1944, **47**, 65.

14692 P

Bartonellosis: A Cause of Severe Anemia in Splenectomized Dogs.

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Although bartonellosis is a common complication following splenectomy of rats, it rarely occurs among splenectomized dogs. We have found only 12 reports in the literature dealing with this disease in dogs including the original report by Kikuth.[1] For this reason, and because we had difficulty in accounting for the development of severe anemia in one of our dogs, it seemed worth while to record the first case in the dog to be recognized in this laboratory. We are not aware of a previous report from the western two-thirds of the United States.

Report of Case. Dog 1 was an apparently healthy female which was being used in a study of the effect of pentobarbital sodium anesthesia on the concentration of hemoglobin before and after splenectomy. On December 3, 1943, the concentration of hemoglobin was 15.3 g per 100 cc of blood. On December 8, 1943, its spleen was removed. The animal apparently recovered from the operation satisfactorily and on December 28, 1943, the concentration of hemoglobin was 13.0 g per 100 cc of blood. By January 24, 1944, the concentration of hemoglobin had dropped to 5.4 g per 100 cc of blood without any obvious reason. Dr. George M. Higgins, who had studied bartonellosis in rats, suggested that bartonellosis might be the cause of the anemia. Examination of stained (Giemsa) smears of blood revealed the characteristic pleomorphic organisms associated with the erythrocytes. The organisms varied from single coccoid forms to beaded and filiform rods and were apparently *Bartonella canis* (Fig. 1). Because of this finding a tentative diagnosis of bartonellosis was made. The concentration of hemoglobin of the dog's blood continued to decrease until on March 9, 1944, it was only 1.8 g per 100 cc. After this there was a spontaneous improvement and by March 30, 1944, the hemoglobin had increased to 7.9 g per 100 cc of blood. However, stained

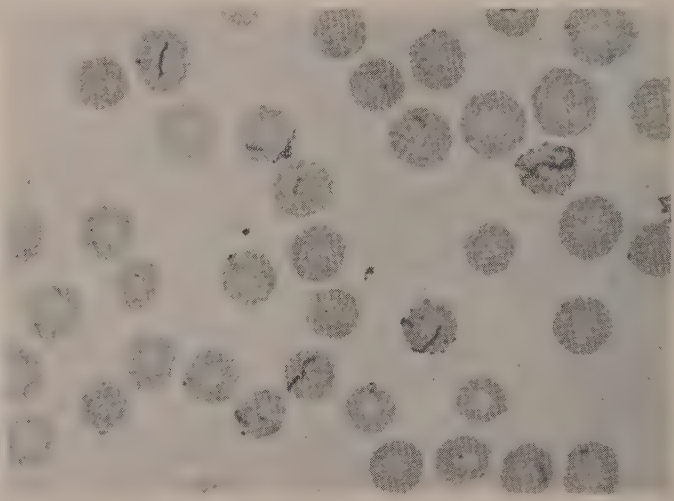

Fig. 1.

Giemsa stained blood smear showing the pleomorphic *Bartonella canis* organisms associated with the erythrocytes.

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[1] Kikuth, W., *Klin. Wchnschr.*, 1928, **7**, 1729.

smears of the blood continued to show *Bartonella canis.*

On February 5, 1944, Dog 2 was inoculated with 5 cc of whole blood taken from Dog 1. On that date the concentration of hemoglobin was 11.6 g per 100 cc of blood. Previous studies of stained smears of the blood of this animal had not revealed *Bartonella canis.* On February 9, 1944, the dog's spleen was removed. Stained blood smears and the concentration of hemoglobin were studied at weekly intervals. On March 3, 1944, the erythrocytes showed Bartonella bodies and by March 30, 1944, the hemoglobin had decreased to 7.4 g per 100 cc of blood. The hemoglobin continued to decrease and on April 20, 1944, the concentration was only 3.2 g per 100 cc of blood.

Dog 3, which had been splenectomized on September 5, 1939, was inoculated with 5 cc of whole blood from Dog 1 on March 3, 1944. The results were similar to those obtained in the case of Dog 2 and need not be recorded in detail.

The demonstration of organisms that had the morphologic characteristics of *Bartonella canis,* the transmission of the infection to other dogs with resultant anemia, and the demonstration of the organisms in their blood confirmed the diagnosis of spontaneous bartonellosis.

Summary. After splenectomy had been performed on a dog severe anemia developed and stained smears of the blood revealed organisms that had the morphologic characteristics of *Bartonella canis.* The disease was transmitted to two other dogs, confirming the diagnosis of bartonellosis.

14693 P

Effect of Intravenous Injection of Trypsin on the Blood Coagulation Time in Hemophilia.

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Suitable quantities of trypsin will reduce to normal, *in vitro,* the prolonged clotting time of hemophilic blood.[1] The intravenous injection of trypsin into a hemophilic patient has never been attempted, chiefly because experiments on animals have shown that trypsin is toxic and causes intravascular coagulation.[2,3]

However, the toxicity of trypsin is greatly reduced by injecting it slowly. Thus, crystalline trypsin*[4] dissolved in 200 to 300 ml of physiological saline was injected intravenously into 3 hemophilic patients and its effect on the coagulation time observed.

The coagulation time was measured at 37°C on duplicate samples of 2 ml of blood in chemically clean test tubes, 9 to 10 mm in diameter, previously rinsed with physiological saline. The end point is reached when the tube can be completely inverted without spilling the blood.[5]

The results (Table I) show that a significant shortening of the coagulation time followed the injection of 300 mg of trypsin in 19 minutes and of 325 mg in 20 minutes respectively in 2 different hemophilic patients. The coagulation time returned to the initial value after one hour. Observation 11 (Table I) shows that no effect on the coagulation time

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[1] Tyson, L. T., and West, R., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 494.

[2] Rocha E Silva, M., *Arch. Path.*, 1942, **33**, 387.

[3] Tagnon, H. J., *J. Clin. Invest.*, in press.

* Obtained from the Plaut Research Laboratories, Bloomfield, N.J. This preparation contains about 80% magnesium sulphate.

[4] Northrop, J. H., *Crystalline Enzymes*, Columbia University Press, 1939, page 135.

[5] Patek, A. J., and Stetson, R. P., *J. Clin. Invest.*, 1936, **15**, 531.

TABLE I.
Effect of Intravenously Injected Trypsin on Clotting Time of Hemophilic Blood.

Observation No.	Subject	Trypsin (mg)	Duration of injection (min)	Clotting time* (min—37°C) 1	2	3
1	SM.	2	33	88	93	
2	SM.	1	3	99	92	
3	SM.	30	28	125	109	
4	Roc.	40	36	136	124	
5	Roc.	60	28	153	166	
6	Roc.	120	45	109	117	
7	SM.	120	22	67	53	
8	SM.	250	20	90	54	88
9	SM	300	19	*92*	*30*	*91*
10	Roc.	325	20	*104*	*24*	*92*
11	T.K.	300	120	69	78	70

* 1. Before injection.
2. Immediately after the end of injection.
3. One hour after the end of injection.

TABLE II.
Effect of Trypsin on Clotting Time of Hemophilic Blood *in Vitro*.

Test tube No.	Trypsin (mg in 0.1 ml of saline)	Hemophilic blood* (ml)	Clotting time (min room temp.)
1	2	2	13
2	2	2	15
3	1	2	8
4	1	2	8
5	0.5	2	8
6	0.5	2	8
7	0.25	2	*5*
8	0.25	2	*5*
9	0.1	2	7
10	0.1	2	9
11	0.05	2	12
12	0.05	2	15
13	0	2	72
14	0	2	93

* From patient T.K.

followed the injection of 300 mg during a period of 120 minutes.

No toxic manifestations, local or general, were observed during or after the injections.

Table II shows that *in vitro* the optimal quantity of trypsin effective on the coagulation time of 2 ml of hemophilic blood was 0.25 mg. A significant effect was also observed with from 1.0 to 0.05 mg. Assuming the blood volume of the patients to be about 5000 ml, there seems to be a definite correlation between the effect of trypsin *in vivo* and *in vitro*. On this basis, a dose of 300 mg of trypsin should be expected to produce an effect *in vivo*.

Larger doses of trypsin were not tried because the toxic dose is probably very near the dose most effective on the clotting time.[3] For the same reason it was felt that normal subjects should not receive injections of trypsin in such doses as produced a distinct shortening of the coagulation time in hemophilic patients.

The action of intravenously injected trypsin, as described here, is of short duration (less than 1 hour) (Table 1). Its therapeutic effectiveness in hemophilia thus does not compare with transfusion of either fresh blood or plasma. Furthermore, the toxicity of trypsin is great and extreme caution is advised in administering it intravenously. However, further investigations may be able to make its use safer and more effective.

14694

Biotin-Pantothenic Acid Interrelationship in Rats Fed Succinylsulfathiazole.

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The feeding of so-called "purified" rations containing sulfaguanidine or succinylsulfathiazole to rats has a marked depressing effect upon growth; an inhibition which can be counteracted by the feeding of liver extracts.[1,2] Daft and co-workers[3] reported that this deficiency syndrome was ascribable in part to a lack of biotin. The biotin deficiency was not progressive, due in all probability to the coexisting lack of folic acid. These findings were confirmed by Nielsen and Elvehjem[4] and by Martin.[5] The only other means of producing biotin deficiency in the rat is either by the feeding of a purified diet containing egg white or a purified ration with casein as the source of protein but supplemented with an avidin concentrate: the deficiency state thus produced progresses and leads to the ultimate death of the animal.

Wright and Welch[6,7] have reported that folic acid and biotin are necessary for the utilization of pantothenic acid by rats receiving purified diets containing succinylsulfathiazole. Animals fed this type of ration gave evidence of pantothenic acid deficiency despite the inclusion of adequate amounts of this vitamin in their diet. Even parenteral administration of pantothenic acid failed to restore the levels of this vitamin in the liver to the normal. West *et al.*[8] observed changes usually associated with a lack of pantothenic acid in rats maintained on a sulfapyridine containing low protein diet. However, the feeding of excess pantothenic acid cured these deficiency manifestations.

In the course of an investigation dealing with the influence of succinylsulfathiazole upon the utilization of pantothenic acid it was noted that signs of biotin depletion were much more marked in rats receiving the pantothenic acid deficient diet than in controls supplied with this vitamin. At a time when the rats receiving pantothenic acid showed only incipient manifestations of biotin deficiency, namely, spectacled eyes and sparse mole-like fur, the rats fed the pantothenic acid deficient diet showed a more advanced state of biotin depletion such as sore mouth, ventral alopecia and encrustations. These animals also had the porphyrin caked whiskers and pattern greying associated with a lack of pantothenic acid. Curative therapy with biotin was initiated at a time when the rats were failing rapidly from a lack of folic acid. Although some improvement was seen, weight losses were continuous and only incipient

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[1] Black, S., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 308.

[2] Welch, A. D., *Fed. Proc.*, 1942, **1**, 171.

[3] Daft, F. A., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

[4] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

[5] Martin, G. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 353.

[6] Wright, L. D., and Welch, A. D., *Science*, 1943, **97**, 426.

[7] Wright, L. D., and Welch, A. D., *J. Nutrition*, 1944, **27**, 55.

[8] West, H. D., Jefferson, W. C., and Rivers, R. E., *J. Nutrition*, 1943, **25**, 471.

TABLE I.

Group	No. rats	Avg gain in wt, g		No. survivors, 42 days	Deficiency signs		
					Biotin		Pantothenic acid
		30 days	42 days		First observed days on test	Severity attained	First observed days on test
Diet H5 (Pantothenic acid deficient)	10	60	70	All	—	—	29
Diet H5S (Pantothenic acid deficient + 0.5% succinylsulfathiazole)	10	32	42	3	16	Marked	24
Diet H5S + 5 μg biotin	8	50	53	All	—	—	30
Diet H5S + 500 μg Ca pantothenate	8	91	97	All	28	Mild	—
Diet H5S + 5 μg biotin and 500 μg Ca pantothenate	10	118	135	All	—	—	—
Diet H5 + 500 μg Ca pantothenate	10	148	202	All	—	—	—

changes were noted in the pelage during the short survival period. Accordingly a prophylactic experiment was designed in order to show the effect of biotin, pantothenic acid, and the combination of both upon the production of the two deficient states.

Methods and Results. Weanling male rats were segregated into 5 dietary groups, each being represented by 8 to 10 animals as indicated in Table I. The pantothenic acid free ration (H5) had the following composition: vitamin free casein (Labco), 24; sucrose, 60; salts (U.S.P. No. 1), 4; hydrogenated vegetable oil, 10; cod liver oil, 2. The diet was supplemented with 1 mg each of thiamin and pyridoxin, 2 mg of riboflavin, 10 mg of inositol and 100 mg of choline per 100 g. In the diet containing succinylsulfathiazole (H5S) 0.5% of the drug replaced an equivalent amount of sucrose. Groups receiving biotin and calcium pantothenate were fed these vitamins daily by stomach tube.

As can be seen from Table I the feeding of the ration deficient in pantothenic acid which contained sulfathiazole had a marked effect in depressing growth. After 6 weeks 7 of an original 10 animals had succumbed. Signs of a mild degree of biotin deficiency, namely, spectacled eyes and sparse mole-like fur, were apparent in this group after only 16 days on test. This condition progressed rapidly to involve the ventral aspects of the head and trunk. Hopping gait was observed in one animal. Signs usually associated with a lack of pantothenic acid, namely, porphyrin deposits on the whiskers and pattern greying, were first observed after 24 days on the regimen. This is at a somewhat earlier period than with the pantothenic acid deficient diet without the sulfonamide.

The daily feeding of 5 μg of biotin evoked only a slight growth response; all animals, however, survived the 42-day test period. The rats were completely free from the syndrome associated with a deficiency of biotin. Moreover, the feeding of biotin not only prevented manifestations of biotin deficiency but also tended to delay the onset of pantothenic acid deficiency. Although achromotrichia was present porphyrin caked whiskers were seen in only one rat. It is of interest to note that Unna[9] did not observe porphyrin caked whiskers in rats receiving 40 μg of calcium pantothenate although greying was not prevented.[10]

When pantothenic acid was fed, changes ascribable to a deficiency of pantothenic acid were not observed. In addition the feeding of the vitamin had a marked growth stimulating effect. This observation indicates that calcium pantothenate can be utilized to some extent, a finding in variance with that reported by Wright and Welch.[6,7] However, in the present experiments succinylsulfathiazole composed 0.5% of the diet whereas in the ration of Wright and Welch, a higher level of the sulfonamide, namely 2%, was employed.

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[9] Unna, K., *J. Nutrition,* 1940, **20**, 565.

[10] Unna, K., Richards, G. V., and Sampson, W. L., *J. Nutrition,* 1941, **22**, 553.

The feeding of calcium pantothenate alone had an attenuating effect upon biotin deficiency as reflected both in the time of onset of changes ascribable to biotin deficiency and in the severity of the lesions.

Rats receiving both biotin and calcium pantothenate were, at the termination of the experiment, completely free from the skin and pelage changes associated with biotin and pantothenic acid deficiencies. The fact that the growth did not equal that observed in the absence of the sulfonamide, could, in all probability, be ascribed to a lack of folic acid and other factors, since it has been shown that growth is restored to the normal by the feeding of folic acid concentrates.

Summary. Biotin deficiency induced in weanling rats by the feeding of a succinylsulfathiazole containing purified ration may be aggravated by superimposing a deficiency of pantothenic acid. The feeding of biotin protected against these changes and, in addition, appeared to lessen the severity of the syndrome associated with a lack of pantothenic acid. Calcium pantothenate, fed prophylactically, was completely protective against deficiency signs usually associated with the absence of pantothenic acid. Furthermore, signs of a mild degree only of biotin depletion were observed and the period required to produce such changes was extended beyond that observed in the absence of pantothenic acid. Rats receiving both biotin and calcium pantothenate were free from signs of both deficiency states.

14695 P

Congenital Malformations of the Eyes Induced in Rats by Maternal Vitamin A Deficiency.*

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It has been repeatedly suggested that congenital blindness in animals may be caused by a maternal nutritional deficiency. Blindness in cattle associated with a constriction of the optic nerve of probable nutritional origin was reported by Moore *et al.*[1] who also reviewed the literature concerning this subject. Hale [2] reported the occurrence of anophthalmos and microphthalmos in pigs whose mothers had been fed a diet deficient in vitamin A. No corresponding findings have been reported in rats. Mason[3] who studied reproduction of vitamin A deficient rats described foetal death, prolonged gestation and difficult parturition but not congenital malformations of the offspring. Cannon[4] also reported failure to produce congenital anomalies in the young of rats whose mothers were depleted of vitamin A.

We have recently observed congenital defects of the eyes in the offspring of rats raised on a diet containing very small amounts of carotene and bred on a diet entirely free of carotene and vitamin A. The preparatory diet (Diet U) fed for approximately 3 months after weaning had the following percentage composition: Ground whole wheat, 74; crude casein (Borden), 15; brewer's yeast (Mead Johnson), 10; sodium chloride C.P., 1. This diet was supplemented by 60 I.U. of vitamin D (Drisdol, Winthrop) and 25 μg of carotene (SMACO) in cottonseed oil every tenth day. This small amount of carotene made possible slow growth and maturation but no storage

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* This work was aided in part by a grant from the Nutrition Foundation, Inc., New York, N.Y.

[1] Moore, L. A., Huffman, C. F., and Duncan, C. W., *J. Nutr.*, 1935, **9**, 533.

[2] Hale, F., *J. Hered.*, 1933, **24**, 105.

[3] Mason, K. E., *Am. J. Anat.*, 1935, **57**, 303.

[4] Cannon, M. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 129.

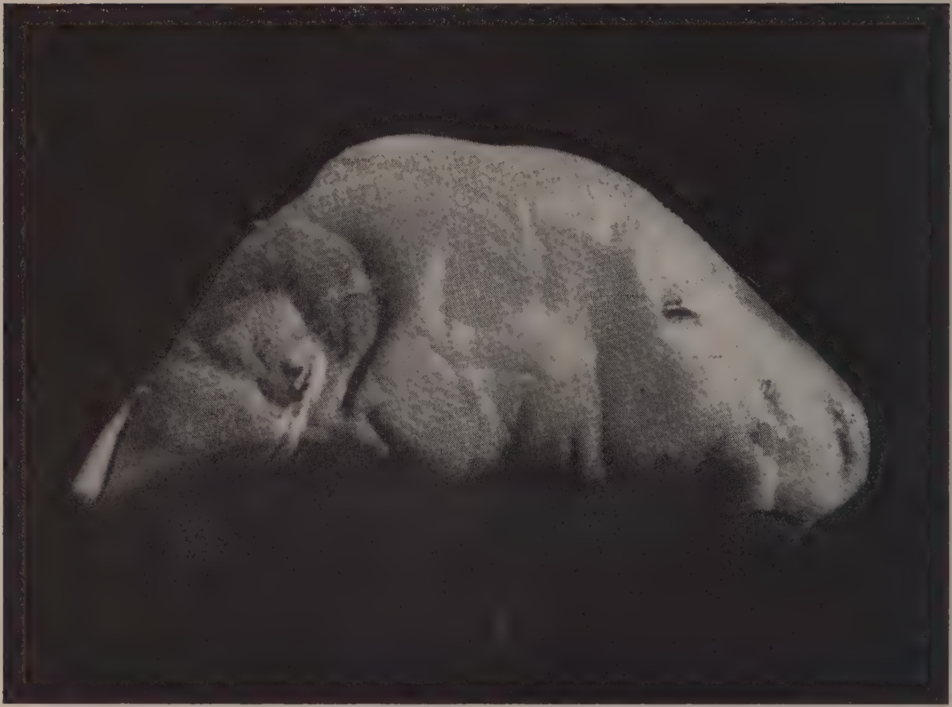

FIG. 1.
Showing abnormal "open eye" in newborn rat.

of the vitamin. If the growth curves of the rats became flat during this preparatory period, which happened about 3 or 4 times, each rat was given 5 g of horse muscle.

The purified diet (Diet W) which was completely free of vitamin A had the following percentage composition: Sucrose, 68; vitamin test casein, 18; vegetable oil, 10; salt mixture,[5] 4. One hundred grams of this diet were supplemented by 0.8 mg thiamine hydrochloride, 0.8 mg pyridoxine hydrochloride, 0.8 mg riboflavin, 1 mg calcium pantothenate, 10 mg niacinamide, and 100 mg choline chloride. This diet was supplemented every tenth day by vitamin D (1 drop of a 1:4 dilution with olive oil of Drisdol), vitamin E (1 drop of a solution of 5 g alpha-tocopherol in 100 ml of olive oil), and vitamin K (1 drop of a solution of 1 mg of 2-methylnaphthoquinone in 5 ml of olive oil).

Twenty female rats of the Sprague-Dawley strain were raised on diet U until they reached a weight of 150-160 g and regular oestrus cycles were established. Then they were placed on the purified diet W and bred to males of the same strain. These males had been fed an adequate diet. Twelve of the females bred as was confirmed by the finding of sperm in the vaginal smears. Within a week the females fed diet W showed marked signs of nutritional deficiency and only three mothers carried their litters to or near term, while nine females resorbed their embryos. The three litters obtained by caesarean sections consisted of 19 dead young, all of which had abnormal eyes.

In 14 of these young the abnormality could be recognized by external examination. Instead of the closed eyes seen in normal newborn rats "open eyes" of a reddish color were seen (Fig. 1). These eyes as well as the closed eyes of 5 of the young were abnormal on histological examination.

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[5] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutr.*, 1937, **14**, 273.

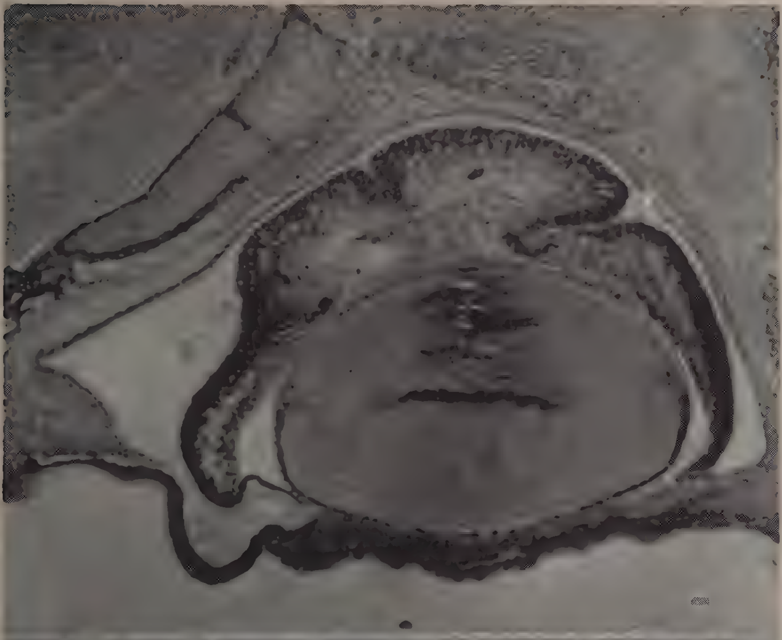

Fig. 2B. Section of an abnormal "open eye." (× 70.)

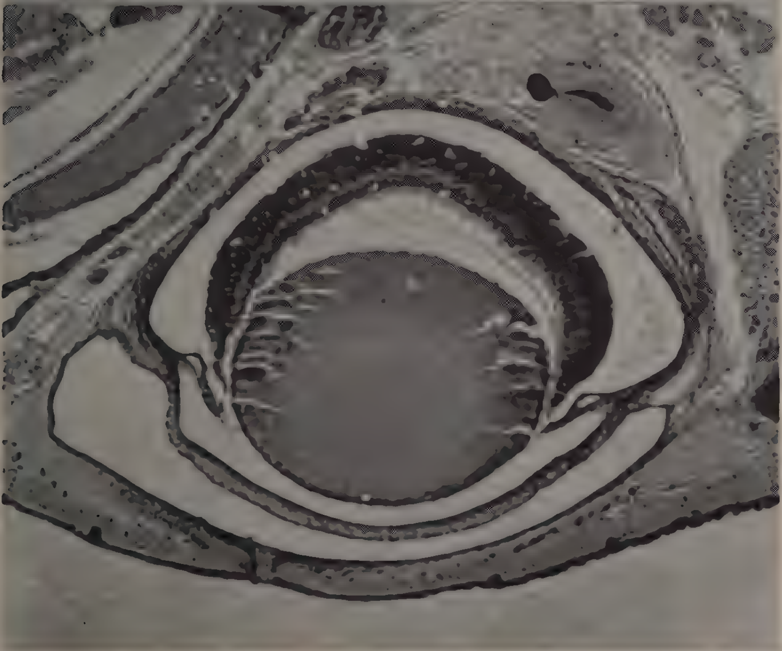

Fig. 2A. Section of the eye of a normal 19-day rat fetus. (× 70.)

Fig. 2A represents the eye of a normal 19-day rat fetus and Fig. 2B an "open eye." In the normal eye the lids, the conjunctival sac, the cornea, the anterior chamber and the iris appear as well defined separate structures and spaces. In the abnormal eye there is no clear differentiation of the lids and the cornea; the anterior chamber is present in a rudimentary form only. In the normal eye the vitreous can be seen between the lens and the retina, while the same space in the abnormal eye is filled with connective tissue. The retina of the abnormal eye is folded and disorganized. In the closed abnormal eyes the lids are fused with the cornea and the anterior chamber appears as a linear space between the thick membrane thus formed and the lens. In several specimens a cleft can be seen in the inferior part of the retina and a strand of connective tissue which penetrates this cleft, spreads out in the space between the lens and the retina. This cleft represents a coloboma of the retina.

As controls 62 females of the same strain were fed diet W supplemented with vitamin A. They had 410 young, none of which showed abnormal "open eyes."

14696

Effect of Dietary Hepatic Injury on Inactivation of Estrone.

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Golden and Sevringhaus[1] observed in the rat that when a functioning ovary was transplanted into the mesentery, estrus did not occur. They concluded that estrogen which was thus routed through the portal circulation had been inactivated by the liver. Failure of the liver to inactivate estrogen has been demonstrated by Biskind and Biskind[2] in rats maintained on a vitamin-deficient diet. These workers found that ovariectomized rats receiving a normal diet showed no vaginal cornification after estrone pellets were implanted in the spleen, while animals on a diet deficient in the vitamin B complex went into estrus within 1 to 3 weeks. The occurrence of estrus was interpreted as an indication of impaired liver function. Estrus could subsequently be made to disappear by the addition of yeast to the diet.

In the liver disorder produced by Biskind and Biskind, no anatomical changes in the organ were observed. The experiments were therefore concerned with functional changes resulting from the avitaminosis. It is easily possible to produce severe hepatic necrosis and cirrhosis by the administration of diets low in protein and high in fat.[3] The following experiments were performed in order to determine whether the hepatic lesions so produced would result in failure of estrogen inactivation and whether evidence of repair could be detected by the disappearance of estrus during treatment. In an additional series of rats, the experiments of Biskind and Biskind with B-complex-free diets were repeated.

Methods. Rats used in the cirrhosis studies were of Sprague-Dawley strain. Those that received the B-deficient diet were of 3 separate strains. The animals weighed approximately 200 g. About 10 days after ovariectomy, 2 γ of estrone were injected in order to test the reactivity of the animal. If reactive, a 5 mg pellet of estrone was implanted in the spleen.* More than half the rats went into estrus immediately thereafter but, in most cases, this lasted only from 2 to 4 days.

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[1] Golden, J. B., and Sevringhaus, E. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 361.

[2] Biskind, M. S., and Biskind, G. R., *Endocrinol.*, 1942, **31**, 109.

[3] György, Paul, and Goldblatt, Harry, PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 492; Webster, Graham, *J. Clin. Invest.*, 1941, **20**, 440; Blumberg, Harold, and McCollum, E. V., *Science*, 1941, **93**, 598; Lillie, R. D., Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1941, **56**, 1255.

* We are indebted to Dr. D. K. Kitchen of Parke, Davis and Co. for the supply of Estrone (Theelin).

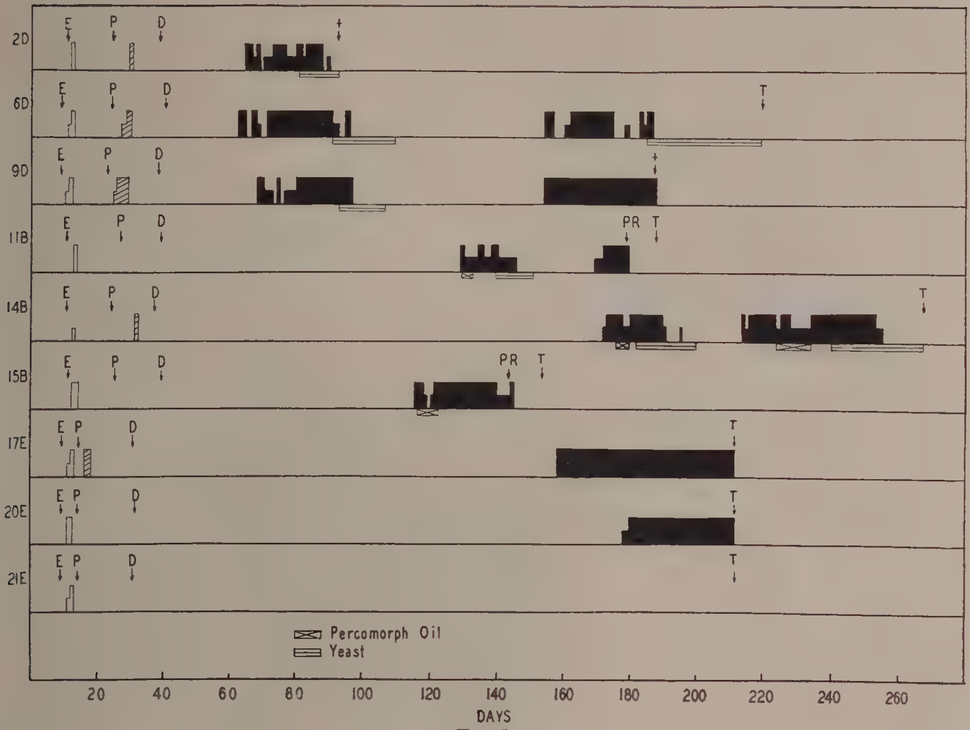

FIG. 1.

Series of rats receiving a diet low in protein and high in fat. The blocked areas represent estrus. Partial estrus is depicted by a reduction in height of the block. Symbols are as follows: E—test dose of estrone; P—insertion of pellet; D—diet started; PR—pellet removed; T—experiment terminated; + — death of animal. The rats were castrated at zero time.

If it persisted longer than 10 days, the animal was discarded. Of the rats originally ovariectomized, 60% fulfilled the necessary conditions to proceed with the experiment. The rate of absorption from the pellets average 11 γ a day.

The experimental diets were as follows:

Cirrhosis Producing Diet.

Casein	8%
Crisco	38%
Sucrose	48%
Cod liver oil	2%
Salt mixture	4%

Vitamin supplement—1 cc daily.[†]

B-complex Deficient Diet.

Casein	18%
Peanut oil	8%
Sucrose	68%
Cod liver oil	2%
Salt mixture	4%

Results. Cirrhosis Diet. Nine rats received the diet. Eight went into estrus within 25 to 150 days, but one animal (21E) did not respond within the period of 180 days during which it was followed. The vaginal response was occasionally erratic during the first few days succeeding its onset, but usually remained constant thereafter. Percomorph oil, 3 drops daily (5000 I.U.), did not abolish the reaction but sometimes decreased it. After the estrus had persisted for 10 to 30 days, 5 rats were treated with Brewer's yeast (Anheuser Busch strain G), added to the basic diet in a concentration of 25%. In all cases, there was disappearance of estrus after an interval of 3 to 15 days. Rat 2D died of unexplained causes shortly after disappearance

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[†] The supplement contained in each cc: Thiamine 20 γ, pyridoxine 20 γ, riboflavin 25 γ, Ca pantothenate 100 γ.

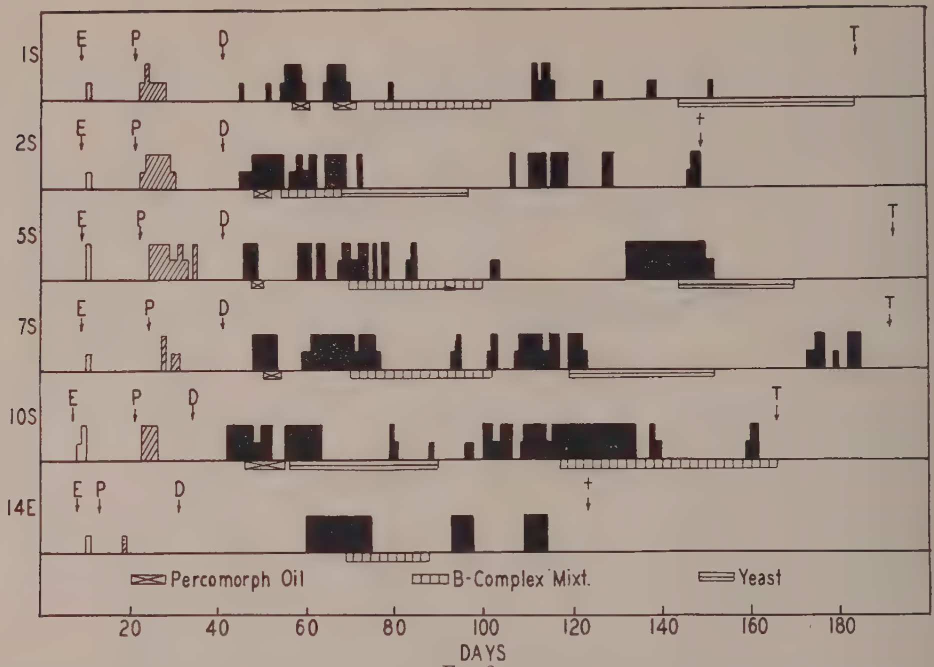

FIG. 2.

The effect of Vitamin B-complex deficiency. The symbols have the same meaning as in Fig. 1.

of estrus. Four of the 5 rats were taken off yeast and estrus again appeared. It was made to disappear a second time in rats 6D and 14B by resumption of yeast therapy. The livers of the rats on the cirrhosis-producing diet showed the usual fatty changes along with necrosis and fibrosis.

B-complex Deficient Diet. A group of 7 hooded rats bred in the laboratory were fed the diet for 23 days without the appearance of estrus in any rat. Nine rats of Sprague-Dawley strain were followed until they either died or showed estrus. Seven out of this group responded with positive smears within 7 to 37 days; however, 3 did not react until they were extremely emaciated and near death. The 2 which failed to respond died after 42 and 56 days respectively. A group of 6 Sherman rats, kindly supplied by M. S. Biskind, promptly and uniformly went into estrus. All became positive within 5 to 14 days, and before any serious ill effects from the diet were evident.

In general, the estrus from avitaminosis B was not as solidly sustained as that following the cirrhosis-producing diet but tended to be interrupted by periods of anestrus. In the rats which responded, estrus could usually be abolished by the administration of 3 drops of percomorph oil daily. It was at first suspected that this was indicative of a false estrus due to vitamin A deficiency. It was proved unlikely that such was the case, however, inasmuch as this dose of oil prevented estrus after the injection of 2 γ of estrone into several reactive castrate rats. Neither cornified nor nucleated cells appeared in the smears. Furthermore, a similar dose of the oil usually caused normal cyclic females to remain in diestrus. It has been reported that vitamin A in large doses prevents vaginal cornification and stimulates the formation of nucleated cells.[4] In the series at hand, the effect was to maintain complete anestrus.

Administration of 1.8 g brewer's yeast daily to 5 rats which went into estrus resulted in total anestrus in 4 of them within 4 to 8 days.

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[4] Sherwood, T. C., Brend, M. A., and Roper, E. A., *J. Nutrition*, 1936, **11**, 592.

The fifth (10S) was treated for 37 days and during this time was for the most part anestrus although there were occasional days on which cornified cells in good number were encountered. Seven rats were treated with 1 cc of the B-complex mixture daily for 20 to 46 days. While on the supplement, these rats showed a tendency to remain in anestrus but the response was for the most part incomplete (Fig. 2). Fifty mg para-aminobenzoic acid daily in addition to the other B vitamins did not improve the response of one rat (10S) showing partial anestrus.

The livers of the untreated rats on the B-deficient diet showed no histological evidence of damage except in one case where a slight fatty change was demonstrable.

Discussion. The impairment of estrone inactivation which was observed in rats receiving a diet low in protein and high in fat may be assumed to have resulted from the accompanying injury to the liver. The onset of functional disability appeared after an interval when severe histological changes are known to make their appearance. It would appear that the method should be useful in the study of experimental cirrhosis. Reversal of the estrus reaction by the administration of large doses of yeast indicates that curative measures may be very nicely assessed in the living animal.

In confirmation of the work of Biskind and Biskind, vitamin B-complex deficiency was found to interfere with hepatic inactivation of estrone. The alteration produced by the B-deficient diet, however, was neither so constant in occurrence nor so solidly sustained as that which followed the cirrhosis-producing diet and the effects of therapy were not so easily judged. There appeared to be a strain difference in response. Sherman rats were very susceptible while a local strain of hooded rats was relatively resistant. Sprague-Dawley rats were intermediate. The B vitamins known to be required by the rat were not adequate for the complete reversal of the effect when given in ordinary maintenance doses. Segaloff and Segaloff,[5] however, in somewhat similar experiments recently reported that large doses of riboflavin and of thiamine were effective.

Summary. Liver damage produced in rats by diets low in protein and high in fat was consistently reflected in the living animal by impairment in ability to inactivate estrone. The curative effect of large doses of yeast could be demonstrated.

Vitamin B-complex deficiency was usually accompanied by a similar but less constant hepatic impairment and without anatomical damage to the liver. There appeared to be a strain difference in susceptibility. A mixture of known vitamins was only partially effective in abolishing the effect of the diet, while yeast was distinctly and more consistently effective.

We are greatly indebted to Miss Ethel Buchwald for technical assistance.

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[5] Segaloff, A., and Segaloff, A., *Endocrinol.*, 1944, **34**, 346.

14697

Spasmolytic Activity of Diethylaminoethyl Esters of Substituted α-Thienylacetic and α-Thienylglycolic Acids.

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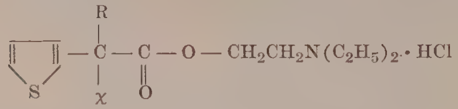
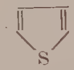
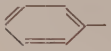
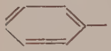
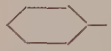
From the Pharmacological Research Laboratory, Frederick Stearns and Company, Detroit, Mich.

During recent years a number of reports have appeared which described the pharmacologic activity of a variety of synthetic products which were developed in an effort to obtain an effective antispasmodic substance that would be relatively free of the undesirable side actions of atropine. These substances may be conveniently divided into two groups: (1) amines, such as Perparine, Cyverine, or Sestron, and (2) basic esters of arylaliphatic

TABLE I.
Antispasmodic Activity.
Rabbit Jejunum



Compound No.	R	χ	Isolated rabbit jejunum		Intact rabbit jejunum		
			acetylcholine*	$BaCl_2$†	Dose—i.v. mg/kg	Response	Duration min
612		H	1-4 M(illion)	1-200,000	0.20-2.00	+, ++	3-45
606		H	5 M	200,000-400,000	0.10-2.00	0, +++	0-90
600		OH	60 M	200,000-400,000	0.05-1.00	++, ++++	10-45
623		OH	80 M	500,000	0.01-0.10	+++, ++++	6-135
β-diethylaminoethyl diphenylacetate‖			1 M	200,000	0.20-2.00	0, +++	0-45
β-diethylaminoethyl cyclohexylphenylacetate‡			3 M	500,000	0.01-0.02	+	Transient
β-diethylaminoethyl fluorene-9-carboxylate§			2 M	200,000	0.50-1.00	++	13-16
Atropine Sulfate			50 M		0.10-0.20	++++	60
Papaverine				200,000			

* Maximum dilution that will cause complete contracture. Usually 1-1M, 2M.
† Maximum dilution that will cause complete contracture. Usually 1-10,000.
‡ Graham and Lazarus.[7]
§ Pavatrine, *cf* Lehmann and Knoefel.[8]
‖ Trasentin.

acids, such as Trasentin or Syntropan. The first group exerts its action primarily on the smooth muscle cell; the second is effective largely by virtue of the ability to diminish or abolish cholinergic activity in organs composed mostly of smooth muscle. It has been previously pointed out by Tainter,[1] Alles and Feigan,[2] Blicke and Zienty,[3] Blicke and Burkhalter,[4] and Warren *et al.*,[5] that the substitution of the thiophene for the benzene ring in various pharmacologically active molecules yielded compounds that are, in a pharmacologic sense, equivalent. Accordingly, Blicke and Tsao[6] have prepared a series of basic esters of substituted α-thienylacetic and α-thienylglycolic acids (α-thienylhydroxyacetic acid) which were tested by us for their antispasmodic activity and toxicity. Chemical formulas of these compounds are indicated in Table I.

Toxicity. Albino mice weighing between 15 and 20 g were used as test animals. They were housed at a relatively constant temperature of 75°F and a relative humidity of 35%. The animals were kept under these conditions for at least 7 days before being used for toxicity determinations. The drugs were dissolved in distilled water, keeping the amount of fluid to be injected at the smallest volume that could be accurately measured. Survival was recorded for 72 hours. The results obtained are shown in Table II. Compound No. 612, the di-α-thienylacetate, was found to be the least toxic (500 mg/kg approximately); No. 600 (phenyl-α-thienylglycolate) the most toxic (131 mg/kg). Saturation of the benzene ring to produce the corresponding cyclohexyl-α-thienylglycolate significantly reduced the toxicity. The corresponding acetate was not available for testing. Graham and Lazarus[7] have reported that basic esters of acetic acid with hydrogenated phenyl groups are considerably less toxic than those containing phenyl groups, but did not indicate the source of their information. However, in their own investigations, the cyclohexyl analog of Trasentin (Trasentin –6H) was found to be somewhat more toxic than Trasentin. The influence of the hydroxyl group of glycolic acid is interesting. The toxicity of compound No. 600 is approximately twice that of the corresponding reduced acid. Similarly, Lehman and Knoefel[8] have reported that diphenylglycolate of β-diethylaminoethanol to be twice as toxic as Trasentin. In view of the above, it is interesting to note that No. 623, the cyclohexyl analog of No. 600, is significantly less toxic than this latter substance.

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[1] Tainter, M. L., *Quart. J. Pharm. and Pharmacol.*, 1930, **3**, 584.

[2] Alles, G. A., and Feigan, G. A., *J. Pharm. and Exp. Therap.*, 1941, **72**, 267.

[3] Blicke, F. F., and Zienty, M. F., *J. Am. Chem. Soc.*, 1941, **63**, 2945.

[4] Blicke, F. F., and Burkhalter, J. H., *J. Am. Chem. Soc.*, 1942, **64**, 477.

[5] Warren, M. R., Marsh, D. G., Thompson, C. R., Shelton, R. S., and Becker, T. J., *J. Pharm. Exp. Therap.*, 1943, **79**, 187.

[6] Blicke, F. F., and Tsao, M. U., *J. Am. Chem. Soc.*, in press.

TABLE II.
Toxicity of Antispasmodic Compounds.
(According to the method of Behrens.)

Compound No.	LD$_{50}$* mg/kg
612	500 approx.
606	260
600	131
623	225
β-diethylaminoethyl diphenylacetate	194
β-diethylaminoethyl cyclohexylphenylacetate	240†
β-diethylaminoethyl fluorene-9-carboxylate	320‡
Atropine Sulfate	240

* Intraperitoneal injection into albino mice.
† Graham and Lazarus.[7]
‡ Lehmann and Knoefel.[8]

Action on the Gastro-intestinal Canal. Antispasmodic action was determined on isolated segments of rabbit jejunum and colon according to the method of Magnus. Contracture was induced by acetylcholine or $BaCl_2$ and the maximum dilution of antispasmodic that would cause 75% relaxation within 2 minutes was determined. The average results obtained are shown in Table I. The glycolates were found to be much more active than the acetates against acetylcholine contractures. No. 623 was found to be the most active, being significantly greater than the corresponding acetate, No. 600, and approximately equal to atropine, when differences in molecular weight are considered (atropine sulfate, M.W. 676.80; No. 623, M.W., 375.95). The activity of No. 623 against $BaCl_2$ induced contractures of the jejunum is about 2.5 times that of papaverine. All were about equally effective against $BaCl_2$ contractures of the colon.

Antispasmodic activity of these substances on the intact intestine of anesthetized rabbits was determined by the method of Barbour. Results are shown in Table I. As above, No. 623, the cyclohexyl-α-thienylglycolate was found to be most effective in reducing tonus. Distinct responses were obtained with doses of 0.01 to 0.10 mg/kg (Fig. 1).

Action on the Uterus. Segments of the isolated rat uterus were tested according to the method of Magnus. Results obtained are shown in Table III. Compounds No. 623 and 600 were found to be more active in relaxing acetylcholine contractures than was No. 606. In a few instances, effects on the motility of the intact uterus were determined by the method of Barbour. Doses of these substances that are effective in relaxing the intact jejunum usually depress uterine motility (Fig. 2).

Other Actions. The effect of these antispasmodic drugs on the vascular response following the intravenous injection of acetylcholine was determined in dogs anesthetized with Na pentobarbital. Acetylcholine was injected in an amount that would cause a 40-60 mm Hg. fall in carotid blood pressure.

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[7] Graham, J. D. F., and Lazarus, S., *J. Pharm. Exp. Therap.*, 1940, **69**, 331.

[8] Lehmann, G., and Knoefel, P. K., *J. Pharm. and Exp. Therap.*, 1942, **74**, 274.

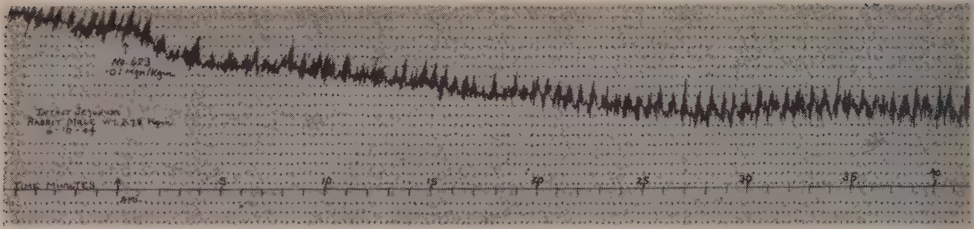

FIG. 1.
Intact rabbit jejunum. At ↑, Comp. No. 623, .01 mg/kg, was injected intravenously.

TABLE III.
Antispasmodic Action on Isolated Colon and Uterus.

Comp. No.	Rabbit colon Acetylcholine (1-1 M)	Rabbit colon $BaCl_2$ (1-10,000)	Rat uterus Acetylcholine (1-10,000)	Rat uterus $BaCl_2$ (1-10,000)
	1-	1-	1-	1-
606	1 M(illion) 2 M	200,000- 500,000	200,000- 500,000	100,000
600	20 M	200,000- 500,000	1 M- 4 M	100,000
623	40 M	500,000	4 M- 20 M	100,000 200,000
β-diethylaminoethyl diphenylacetate	500,000	200,000- 500,000	200,000	200,000
β-diethylaminoethyl fluorene-9-carboxylate	1 M	200,000- 500,000	500,000- 1 M	100,000
Atropine Sulfate	40 M		4 M- 20 M	
Papaverine		500,000		

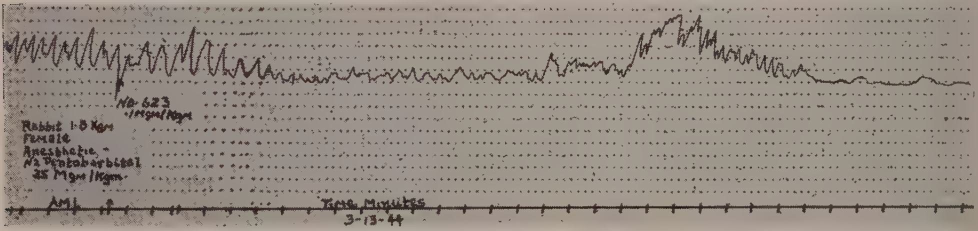

FIG. 2.
Intact rabbit uterus. At ↑, Comp. No. 623, .1 mg/kg, was injected intravenously.

No. 623, in intravenous doses of 0.025-0.10 mg/kg, reduced the acetylcholine fall by 78-100%; No. 600 in the same amounts 56-80%; Nos. 606 and 612 in amounts up to 1 mg/kg either had no effect or caused only a small and transient reduction. With compound No. 623, some effect on the vascular response to acetylcholine was noted for 53 to more than 150 minutes; with No. 600 for 17 to 146 minutes. Comparisons with the action of atropine sulfate suggest that No. 623 is probably less active, and No. 600 definitely less active, in depressing the vascular response to acetylcholine.

TABLE IV.
Vascular and Mydriatic Activity of Antispasmodics.

	Vascular action			Mydriasis		
Comp. No.	Dose, mg/kg	Max. depression,* %	Duration depression, min.	Conc. %	Mydriasis	Duration, hr
612	.5 -1.0	0- 30	6- 20	1.0		—
606	.25 -1.0	0- 28	0- 9	1.0	Maximum	2
600	.05 -2.0	56- 80	17-146	1.0	”	1¼-4½
623	.025-2.0	78-100	53-150	.1	”	8-24
β-diethylaminoethyl diphenylacetate	.5 -1.0	0- 15	0- 5	1.0	Slight	1½
β-diethylaminoethyl cyclohexylphenyl acetate	1.0	—	14	More active than Trasentin†		
β-diethylaminoethyl fluorene-9-carboxylate				1.0	Maximum	8
Atropine Sulfate	.01 - .025	50-100	35-165	.1	”	8-24

* % reduction in response to acetylcholine.
†Graham and Lazarus.[7]

The effect of the antispasmodic compounds on salivary secretion was determined in rabbits by the method described by Graham and Lazarus.[7] Pilocarpine in doses of 20-50 mg was subcutaneously injected in the flank region of various rabbits and after 15 minutes, a spasmolytic amount of the antispasmodic drug was injected into the marginal ear vein. The effect on the subsequent secretion of saliva was determined and compared with that secreted in a control period without the antispasmodic. Compound 612 was not used here because of an insufficient amount of the substance. No. 606 was without any definite effect whereas Nos. 600 and 623 definitely depressed, but did not abolish, salivary secretion.

Mydriatic and local anesthetic activity were determined in albino rabbits. The drug was instilled into the palpebral sac, the eyelids held closed for 1 minute then released and the excess fluid allowed to drain away. The size of the pupil was determined under direct illumination. Mydriasis obtained is shown in Table IV. As in previous tests, No. 623 was found to be the most potent substance. Maximum mydriasis was observed when these substances were given in dilutions of 0.01%. In 1% solution, No. 612 was without effect and No. 606 induced mydriasis lasting 2 hours. Compound No. 600, as a 1% solution, caused mydriasis which lasted for 1-5 hours. Anesthesia was determined by touching the cornea with a blunted pencil. Both Nos. 606 and 600 induced moderate anesthesia of the cornea. A 1% solution produced anesthesia lasting 12 to 60 minutes.

Summary. The β-diethylaminoethyl esters of some substituted α-thienylacetic and α-thienylglycolic acids have been investigated in the experimental animal and found to possess important antispasmodic properties. The α-thienylglycolic acid esters are significantly more active than the former but also have greater effect on other cholinergically controlled structures, thus resembling atropine more than do the acetates. The antispasmodic activity of the cyclohexyl-α-thienylglycolate (No. 623) is comparable to that of atropine; the phenyl-α-thienylacetate (No. 606) appears to be somewhat more effective than β-diethylaminoethyl diphenyl acetate and equal to diethylaminoethyl fluorene-9-carboxylate. Compound No. 606 is relatively free from undesirable actions on other autonomic organs. Except for the phenyl-α-thienylglycolate (No. 600), toxicity of these substances is not unfavorable.

14698

Distribution of Bone Marrow, Bone, and Bone-Ash in Rabbits.

Albert A. Dietz. (Introduced by Bernhard Steinberg.)
From the Toledo Hospital Institute of Medical Research, Toledo, O.

Although bone marrow is an important organ, few quantitative studies of it have been made. In the investigations being conducted in this laboratory, a knowledge of distribution of bone marrow in rabbits became desirable. Nye,[1] using a modification of the method of Wetzel,[2] found the total marrow in 2 rabbits to be equal to 2.00 and 2.59% of the body weight. Similar results have been obtained for dogs[3] and newborn infants.[4] In the latter case, one-third of the marrow was found in the skull. The above workers removed the soft tissues by maceration, extracted the fat of the whole bone with ether, and filled bone marrow cavities with agar, gelatin, or Woods metal. The volume of the whole bone was then measured by displacement of water and the volume of the bony structure calculated from the weight and density of bone. The bone marrow volume constituted the difference between these two values. This method represented the bony structure to contain marrow since maceration and extraction leave it porous. It also assumed that the medullary cavities were filled to the same extent that they previously contained marrow. These errors are probably compensated when the marrow of the whole animal is calculated but for individual bones the results are erroneous.

Mechanik[5] determined the bone marrow content of individual bones of 13 cadavers. He considered the bone marrow to be the difference in weight of fresh and macerated bone, after correcting for the loss of bone tissue. He found the total marrow to equal 3.4-6.5 (average 4.6) % of the body weight, and recorded the bone marrow content of individual bones in numerous tables.

Neither of the above methods can be applied readily to the determination of the marrow content of individual bones of rabbits. Advantage was taken of the large difference in the ash content of bone and bone marrow. From the ash analyses of the whole bone, marrow-free bone and bone-free marrow, the quantity of bone and bone marrow present can be calculated. Since the weight of the whole bone, a, equals the sum of the weights of the marrow, x, and the marrow-free bone, y, and the weight of the ash of the whole bone, b, is equal to the weight of the ash of these components, 2 equations in 2 unknowns are obtained: $x + y = a$, and $cx + dy = b$. In the latter c and d are the grams of ash per gram of marrow and marrow-free bone, respectively. Solving these simultaneous equations for x, one obtains:

$$x \text{ bone marrow} = \frac{ad - b}{d - c} \quad \text{(I)}$$

The bone tissue then is the difference between the weights of the whole bone and the bone marrow, or its weight can be obtained directly by solving the equations for y. The ash of the marrow-free bone was determined for as many bones as lent themselves to the method. Because of the low ash content of the bone marrow, representative analyses were used in the calculations. Its ash content can be neglected without introducing a significant error, *i.e.*, $c = 0$. Theoretically, one can use constituents other than ash for the determination of the marrow content of individual bones, but the differences in the analyses of bone and marrow are not so marked nor so uniform.[6] Since the bone and bone-ash are determined simultaneously with the bone marrow, their distribution is included in the tables.

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TABLE I.
Distribution of Bone Marrow, Bone, and Bone-ash in 5 New Zealand Rabbits.

	Marrow*		Bone*		Whole bone*		Bone-ash*	
	Range, %	Avg, %	Range, %	Avg, %	Range, %	Avg, %	Range, %	Avg, %
Ossa Coxæ	8.32-10.04	9.19	5.57- 7.12	6.61	7.44- 7.89	7.61	6.32- 6.90	6.65
Femora	14.08-19.87	17.11	9.39-10.43	9.95	11.34-13.86	12.57	10.51-11.53	11.06
Tibiæ	9.73-12.61	11.54	8.86- 9.71	9.18	9.96-10.42	10.04	9.61-10.36	9.94
Fibulæ	0.26- 0.41	0.34	0.30- 0.42	0.36	0.34- 0.39	0.36	0.34- 0.42	0.39
Tarsi and Patellæ	3.32- 4.18	3.58	3.27- 3.69	3.43	3.29- 3.68	3.48	3.16- 3.82	3.45
Metatarsals	2.34- 4.10	3.15	3.65- 3.83	3.75	3.20- 3.93	3.53	3.47- 3.88	3.72
Other bones of pedes	1.51- 2.11	1.74	2.14- 2.48	2.22	1.88- 2.17	2.03	2.06- 2.31	2.22
Total hind legs	44.08-49.96	46.65	34.07-36.64	35.51	38.21-40.18	39.61	36.19-38.72	37.43
Scapulæ	2.58- 2.94	2.76	2.68- 3.52	3.18	2.80- 3.28	3.04	2.84- 3.51	3.16
Humeri	7.41- 9.18	8.46	4.48- 5.67	5.31	5.91- 7.07	6.47	5.36- 6.20	5.75
Ulnæ	2.12- 2.90	2.57	2.68- 3.17	2.89	2.68- 2.88	2.77	2.81- 3.11	2.91
Radii	1.57- 1.99	1.83	1.80- 2.06	1.95	1.75- 2.03	1.91	1.86- 2.07	1.96
Manus	1.54- 2.56	2.04	2.30- 2.92	2.62	2.18- 2.77	2.39	2.23- 2.70	2.53
Total fore legs	16.69-18.47	17.67	14.92-16.71	15.95	16.04-17.05	16.59	15.55-17.15	16.31
Ribs	4.47- 6.08	5.25	5.07- 6.21	5.82	4.82- 6.10	5.62	4.63- 5.65	5.34
Sternum and claviculæ	0.72- 0.94	0.86	0.38- 0.54	0.48	0.58- 0.69	0.62	0.36- 0.50	0.45
Total chest	5.32- 6.95	6.11	5.45- 6.75	6.31	5.40- 6.78	6.24	4.99- 6.15	5.80
Skull	5.55- 8.27	7.14	13.98-17.35	15.44	11.41-13.24	12.37	13.00-16.03	14.75
Mandibulæ	0.95- 2.15	1.45	5.19- 7.79	6.08	3.87- 5.45	4.36	5.38- 7.86	6.23
Hyoid	0.08- 0.13	0.09	0.04- 0.06	0.05	0.05- 0.07	0.06	0.04- 0.11	0.08
Total head	6.73- 9.92	8.68	19.49-25.20	21.57	15.33-18.76	16.79	18.70-24.00	21.06
Vertebræ, Cervical	2.21- 3.72	2.93	3.80- 4.69	4.16	3.30- 4.15	3.72	3.52- 4.30	3.81
’’ Thoracic	5.08- 6.33	5.59	5.41- 6.00	5.61	5.29- 5.91	5.61	4.84- 5.48	5.14
’’ Lumbar	8.26- 9.63	9.00	8.29- 8.96	8.72	8.28- 9.25	8.83	7.90- 8.66	8.36
’’ Sacral	2.05- 3.08	2.58	1.33- 1.77	1.52	1.59- 2.20	1.90	1.26- 1.69	1.46
’’ Caudal	0.62- 1.16	0.80	0.57- 0.72	0.66	0.59- 0.90	0.72	0.54- 0.71	0.63
Total vertebræ	19.27-23.28	20.89	20.26-21.41	20.66	19.89-21.59	20.78	18.87-20.00	19.40

* % of total.

Experiments. Five adult New Zealand rabbits of different stocks were used. The jugular veins were severed and the animals bled to remove as much blood from the marrow as possible. The dead animals were stored in a refrigerator, and only a few bones were removed at a time for dissection. To prevent loss of moisture, the bones were kept moist with saline during dissection. In cases of duplicating bones, those from the right side were used for analyses of the whole bone. Marrow-free portions of bone and bone-free marrow from the corresponding bones of the left side were analyzed separately. Where there were no corresponding bones, *e.g.*, vertebræ, a marrow-free portion of the bone was analyzed separately. The remaining portion was also ashed, and the sum of the ash of the two parts gave the ash content of the whole bone. For a few bones, *i.e.*, manus, caudal vertebræ, sternum, the analysis of closely related marrow-free bone had to be used in the calculations. All bones, especially those of the head were carefully dissected to remove all non-marrow soft tissue.

The samples were weighed, dried at 110°C, and ashed at 700-800°C. The marrow content was calculated from equation (I). The ash of the marrow was found to be 0.4 to 0.6% and 0.5% was used for the calculations. The results of the calculations are shown in Table I.

Results. These experiments show that the bone marrow of adult rabbits constitutes about one-third of the weight of the skeleton, or about 2.2% of the total body weight. These

TABLE II.
Amount of Bone Marrow and Other Organs in Individual Rabbits.

		Rabbit No.						
Organ		1 ♀	2 ♂	3 ♂	4 ♂	5 ♀	Avg	Avg dev.
Body wt	g	1740	2050	2100	3100	3220		
Liver	g	91.0	65.2	66.6	71.1	83.3		
	%*	5.23	3.18	3.17	2.29	2.58	3.29	0.78
Spleen	g	0.81	1.24	1.64	0.92	0.72		
	%*	0.047	0.060	0.078	0.030	0.022	0.047	0.017
Adrenals	g	0.27	0.14	0.21	0.23	0.25		
	%*	0.016	0.007	0.010	0.007	0.008	0.010	0.003
Marrow	g	48.3	37.3	48.3	62.4	61.0		
	%*	2.78	1.82	2.30	2.01	1.89	2.16	0.30
Bone	g	67.8	75.8	85.6	109.9	103.5		
	%*	3.90	3.69	4.07	3.54	3.22	3.78	0.36
"Whole bone"	g	116.2	113.1	133.9	172.4	164.4		
	%*	6.68	5.51	6.37	5.55	5.11	5.84	0.54
Whole bone-ash	g	36.3	43.1	46.7	63.7	60.7		
	%*	2.08	2.10	2.23	2.06	1.88	2.07	0.08

* % of body wt.

values are in agreement with those found by Nye.[1] Comparing it with other organs, the bone marrow is roughly equal to two-thirds the weight of the liver (53 to 87%), and 50 times (30 to 86) the weight of the spleen, and 250 times (174 to 288) the weight of the adrenals. Correlation with the body weight is unsatisfactory because of the individual differences in body fat content. As an example, Rabbits No. 4 and 5 (Table II) had large fat depots.

Although individual animals showed differences in the total bone marrow, the marrow for each of the bones was distributed in constant proportions to the total. Almost half of the bone marrow was found in the hind legs (including the ossa coxæ). The head contained the smallest amount in proportion to its bone content. The variation in the distribution in the vertebræ was slightly accentuated since one rabbit had only 6 cervical and another only 6 lumbar vertebræ and the number of caudal vertebræ varied from 15-18. The distribution of bone and bone-ash differed from that of the marrow, chiefly because of the relatively large amount of inorganic matter in the skull and mandibles.

From the values given in Table I, the amounts of active and inactive marrow can be estimated. The actual amounts depend upon the age and condition of the animal, and as to whether the partly active marrow is considered active or inactive. If the marrow of the pedes, manus, and caudal vertebræ is classified as inactive, 85-90% of the total marrow is active. Nye[1] estimated the active bone marrow in rabbits as 75%.

Conclusions. A study of distribution of bone marrow in rabbits showed 47% of the marrow in the hind legs, 18% in the fore legs, 6% in the ribs, sternum, and clavicles, 9% in the head, and 20% in the vertebræ. The distribution of bone marrow, bone and bone-ash in the individual bones is recorded in the tables.

14699 P

Restoration of Blood Volume Following Standard Sublethal Hemorrhage.

A. T. Miller, Jr., and Maxine Dark. (Introduced by John H. Ferguson.)

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The restoration of a normal circulating blood volume is the central problem in recovery from shock or hemorrhage. It has long been recognized that accessory factors may significantly affect the ability of the organism to restore a reduced blood volume; in many cases these factors may precipitate irreversible circulatory failure. The majority of experimental studies on shock and hemorrhage have utilized technics which produce death in untreated animals. Since the primary agent (hemorrhage, trauma, burning, or scalding) is sufficient in itself to produce death, the operation of accessory factors is difficult to evaluate. For this reason, we have adopted a standard hemorrhage of one-fourth the blood volume as the test procedure; recovery invariably occurs and restoration of blood volume follows a fairly uniform time course. The quantitative effect of accessory factors, whether favorable or unfavorable, may be estimated from alterations in the time curve of blood volume restoration.

Experimental. Normal, unanesthetized adult dogs were used. Blood volume was calculated from simultaneous values for plasma volume and hematocrit cell volume (due to uncertainty concerning the reliability of cell volume determinations in shock and hemorrhage, the results are expressed directly in terms of plasma volume). Plasma volume was determined by the dye dilution method[1] and hematocrit cell volume by centrifugation in Wintrobe tubes (1 hour at 3000 r.p.m.).

Each animal was standardized in the following manner. The normal dye disappearance curve was established from duplicate tests 1 week apart. The standard plasma volume recovery curve was constructed from the average of 2 or more experiments in which plasma volume was determined prior to, and at intervals for 4 hours following a standard hemorrhage. Post-hemorrhage plasma volume values were calculated by reference of the determined dye concentration values to the normal dye disappearance curve. Plasma volume, in percent of normal, was plotted against time in the plasma volume recovery curves. In a few experiments, animals were placed in a warm room (38-44°C) or in a cold room (4-7°C) immediately following hemorrhage to study the quantitative effect of heat and cold on the rate of plasma volume restoration.

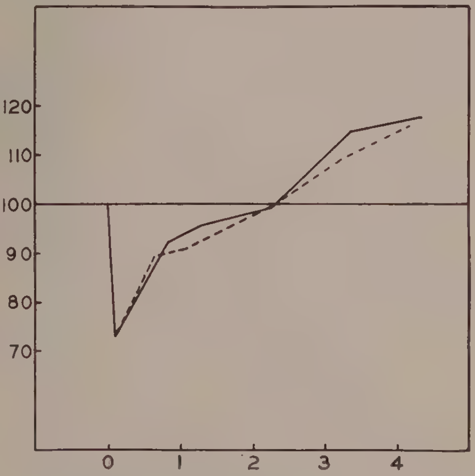

Fig. 1.

Post-hemorrhage plasma volume curves (duplicate experiments on a single animal). Ordinate: plasma volume in percent of normal. Abscissa: time in hours.

Results and Discussion. Fig. 1 shows the results of 2 experiments on the same animal and indicates the high degree of reproducibility possible under controlled conditions. In Fig. 2, the plasma volume recovery curves of 9 experiments on 5 dogs are superimposed. The variation from animal to animal, though small, is sufficient to recommend the use of each animal as its own control unless large numbers of animals are used.

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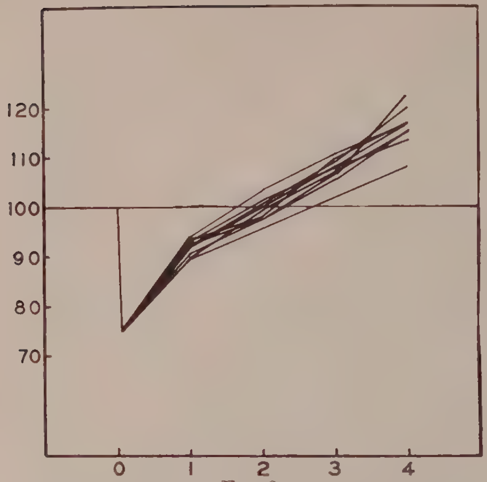

FIG. 2.

Superimposed post-hemorrhage plasma volume curves of 9 experiments on 5 animals. Ordinate: plasma volume in percent of normal. Abscissa: time in hours.

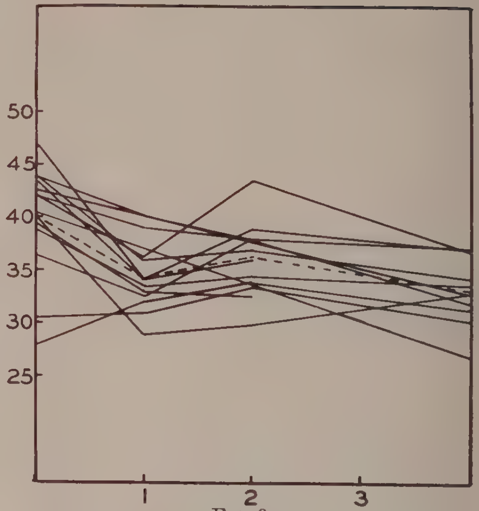

FIG. 3.

Post-hemorrhage changes in hematocrit cell volume. Ordinate: hematocrit cell volume in percent. Abscissa: time in hours.

An analysis of typical curves reveals several points of interest. (1) From 60 to 70% of the plasma lost in hemorrhage was restored during the first hour. (2) Complete restoration of plasma volume (but not of total blood volume) occurred, in the majority of experiments, in 120 to 140 minutes. This is a much more rapid recovery than that reported for the human,[2] but is in accord with the results of similar experiments on the dog[3] and rabbit.[4] (3) The recovery rate decreased during the second hour (presumably due to protein dilution) but again increased during the third and fourth hours in the majority of experiments (the explanation for this secondary increase is uncertain and is being investigated).

The hematocrit cell volume changes following hemorrhage are shown in Fig. 3. The general trend is indicative of hemodilution, but in 9 of 14 experiments hemoconcentration occurred during the second hour. The time relations make it difficult to attribute this result to release of stored cells, and emphasize the danger of relying too heavily on hemodilution as an accurate index of the rate of restoration of fluid to the blood stream.

Preliminary studies have been made on the quantitative effects of heat and cold on the rate of plasma volume restoration. Fig. 4

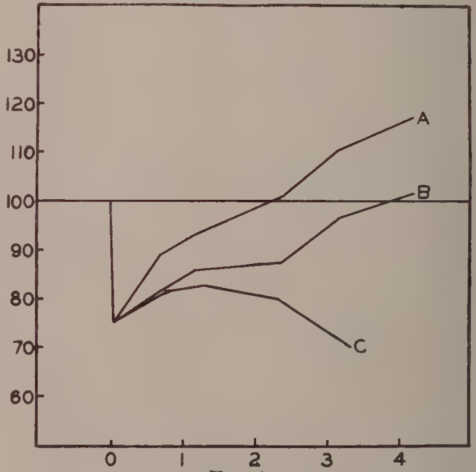

FIG. 4.

Post-hemorrhage plasma volume curves on a single animal (dog number 6). Curve A: normal. Curve B: cold room, temperature 6-7°C. Curve C: warm room, temperature 38-44°C (death occurred after 3 hours' exposure). Ordinate: plasma volume in percent of normal. Abscissa: time in hours.

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illustrates the retarding influence of both heat and cold on recovery rate. The far more drastic effect of heat is in accord with the decreased survival time and increased mortality rate reported[5,6] under similar conditions in hemorrhagic shock. The retarding influence of cold indicates that the explanation for the increased survival time resulting from exposure to cold in hemorrhagic shock[5,6] must be sought elsewhere (it is possible that the effect of cold may depend on intensity, and that milder degrees of cooling may exert a beneficial effect).

Work in progress is designed to quantitate the influence of environmental temperature, anesthesia, anoxia, fluid balance, liver damage, and similar accessory factors on the time course of plasma volume recovery after standard hemorrhage.

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14700

Sensitivity of Various Serological (Lancefield) Groups of Streptococci to Penicillin.*

Robert F. Watson (with the technical assistance of Mary Elizabeth Gardner.)
(Introduced by Homer F. Swift.)

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Penicillin is now being made available in increasing quantities for the treatment of patients. This agent has been demonstrated to be of unquestionable value in the treatment of a number of human infections and to be active against an even larger number of infectious agents in experimental animals and *in vitro*. In general the effectiveness of penicillin in the treatment of infections *in vivo* is closely paralleled by its activity against the same infectious agents *in vitro*.

In previous work[1–5] dealing with the action of penicillin on streptococci *in vitro* these microörganisms have generally been classified either by their fermentation reactions or by the appearance of their colonies on blood agar. The first method is long, difficult, and often yields inconclusive results; and the second is inadequate in that each group contains a large number of antigenically unrelated strains. The Lancefield method of classifying streptococci into serological groups, based on antigenic structure, is now widely used; and groups A through N include most of the strains which have been shown to be pathogenic for man and lower animals.[6,7] In a recent report Dawson *et al.*[8] found strains of streptococci belonging to groups B, C, D, F and H to vary in their sensitivity to penicillin and to be equally as sensitive to ⅛th as sensitive as a group A strain (C 203MV).

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* This article has been released for publication by the Division of Publication of the Bureau of Medicine and Surgery of the U. S. Navy. The opinions and views set forth in this article are those of the writers and not to be considered as reflecting the policies of the Navy Department.

The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

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[8] Dawson, M. H., Hobby, G. L., and Lipman, M. O., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 101.

Although strains belonging to group A account for perhaps 90 to 95% of all human streptococcal infections, groups other than A are occasionally responsible for infections in man; and group B and C strains comprise the chief pathogens for animals. It is also well known that strains belonging to certain groups, namely, B, C, D, H, K, and N are often nonhemolytic or produce only alpha hemolysis; and at times even some group A strains are found to be indifferent or methemoglobin producers under aerobic conditions.

In the present study representative strains of serological groups A through N have been tested *in vitro* for their relative sensitivity to penicillin; and in addition, attempts have been made to demonstrate the presence of a penicillinase or inhibitor for penicillin in a resistant group D strain.

Materials and Methods. Many of the strains of streptococci used had been recently isolated from human infections; the remainder were from stock cultures which had been preserved in the dried state.[†]

For testing the sensitivity of the various strains to penicillin the dilution method described by Rammelkamp[9] was employed. The penicillin was diluted in broth; and to each dilution, 0.5 cc of an appropriate suspension, generally containing between 1,000 and 10,000 streptococci, was added. After 15 to 18 hours incubation at 37°C subcultures were made on blood agar plates which were then incubated overnight. The highest dilution which completely inhibited growth was taken as the end point. This technic is obviously subject to the usual error involved in dilution methods.

The acetone-ether extraction method described by Harper[10] was used to test for the presence of a penicillin inhibitor. The acetone-ether extracted cells were suspended in broth so that each 0.3 cc of the suspension contained 2 mg of dried bacteria. This amount was then added to serial dilutions of penicillin and incubated at 37°C for one hour; then 0.5 cc of culture containing 1,000 to 5,000 cells of a group A strain, C 716, was added to test for the presence of penicillin. The filtrate of a broth culture was also tested for the presence of an inhibitor. A strain of *E. coli* was used as a positive control.

Results. The results of the tests for sensitivity to penicillin of representative strains belonging to the different serological groups (A through N) are presented in Table I. There was very little variation in sensitivity among the strains within any single group. The size of the inoculum, moreover, could be varied within wide limits (100 to 1,000-fold) without changing the amount of penicillin required to inhibit growth completely. Group M strains were the most sensitive and required only 0.0039 Oxford units to sterilize an inoculum containing 25,000 to 35,000 cells. Strains belonging to groups A, C, G, H, and L required 0.0078 to 0.0313 units to kill 1,000 to 10,000 bacteria. Growth of all the group A strains and most of those belonging to the other members of these groups were completely inhibited by 0.0156 units. Strains belonging to groups B, E, F, K, and N were slightly more resistant and generally required 0.0625 units to sterilize an inoculum containing 1,000 to 10,000 cells. Group D strains, which required 2.0 to 5.0 units to kill a similar number of bacteria, were most resistant to the action of penicillin.

Since group D strains were relatively resistant to penicillin, it was of interest to determine whether they produce a substance inhibitory to the action of the drug. The results of tests made with acetone-ether extracted cells and filtrates of broth cultures of strain H 69D5 and a control strain of *E. coli* are recorded in Table II. With this technic a penicillin inhibitor could not be demonstrated in either the cells or filtrate of the culture H 69D5. On the other hand, an inhibitor for penicillin, which was inactivated or destroyed at a temperature of 65°C, was demonstrated in both cells and filtrate of the broth culture of *E. coli*.

Summary and Discussion. The tests re-

[†] The author acknowledges his indebtedness to Dr. R. C. Lancefield for many of these strains.

[9] Rammelkamp, C. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 95.

[10] Harper, G. J., *Lancet*, 1943, **2**, 569.

TABLE I.
Results of Tests for Sensitivity of the Various Serological Groups of Streptococci to Penicillin.

Strain	Group	Type	Source	Inoculum No. bacterial cells	O.U.* inhibiting growth
C 716	A	Undet.†	Dr. C. S. Keefer	100-200	0.0156
"	A	"	" " " "	1,000-2,000	0.0156
"	A	"	" " " "	10,000-20,000	0.0156
1RSC169	A	30	Rockefeller Hospital, Rheumatic Fever, throat	1,000-2,000	0.0156
" 173	A	36	Rockefeller Hospital, Rheumatic Fever, throat	5,000-8,000	0.0156
" 174	A	19	Rockefeller Hospital, Rheumatic Fever, throat	3,000-5,000	0.0156
" 176	A	17	Rockefeller Hospital, Rheumatic Fever, throat	3,000-5,000	0.0156
" 178	A	19	Rockefeller Hospital, Rheumatic Fever, throat	2,000-4,000	0.0156
" 165	A	24	Rockefeller Hospital, Rheumatic Fever, throat	1,000-2,000	0.0156
" 181	A	10-12	Rockefeller Hospital, Acute Pharyngitis and Mastoiditis	3,000-5,000	0.0156
C 203	A	3	Scarlet Fever, Dr. A. R. Dochez	5,000-8,000	0.0078
T 1	A	1	Strain SF 130/2, Dr. F. Griffith	3,000-5,000	0.0156
T 1/72/0	A	1	" " " after 72 mouse passages	4,000-6,000	0.0156
T 12	A	12	" " 42, Dr. F. Griffith	3,000-5,000	0.0156
T 12/36/0	A	12	" " " after 36 mouse passages	3,000-5,000	0.0078
22RS72	A	24	Rockefeller Hospital, Rheumatic Fever, throat	4,000-6,000	0.0156
C 98/88/0	A	24	Strain 22RS72 after 88 mouse passages	2,000-4,000	0.0078
1RSC116	A	3	Rockefeller Hospital, Scarlet Fever	2,000-4,000	0.0078
D 136 A	B	III	Black 771, Subacute Bact. Endocardtis, Dr. R. M. Fry	4,000-5,000	0.0625
" " B	B	II	Brownett 96, Puerperal Sepsis, Fatal, Dr. R. M. Fry	500-1,000	0.0625
" " "	B	"	Brownett 96, Puerperal Sepsis, Fatal, Dr. R. M. Fry	5,000-10,000	0.0625
" " "	B	"	Brownett 96, Puerperal Sepsis, Fatal, Dr. R. M. Fry	50,000-100,000	0.0625
" " C	B	III	Cole 106, Puerperal Sepsis, Fatal, Dr. R. M. Fry	5,000-10,000	0.0625
" " D	B	Ia	SHI Shipley, Puerperal Sepsis, Fatal, Dr. R. M. Fry	5,000-10,000	0.0625
V 9	B	II	Bovine	5,000-10,000	0.0625
16RS72	C		Rockefeller Hospital, Rheumatic Fever, throat	2,000-3,000	0.0156
1RSC73	C		Rockefeller Hospital, Rheumatic Fever, throat	1,000-2,000	0.0156
D 181	C		Strain No. 4 Guinea Pig, Dr. C. V. Seastone	100-200	0.0313
"	C		" " " " " " " " "	1,000-2,000	0.0313
"	C		" " " " " " " " "	10,000-20,000	0.0313
C 519	C		" " 1890, Human throat, Dr. F. Schwentker	5,000-10,000	0.0078
K 150 B	C		Bovine Mastitis, Dr. P. R. Edwards	4,000-8,000	0.0156
J 148 D	C		Monkey, Normal throat, Dr. E. E. Terrell	3,000-6,000	0.0078
D 178 A	D		Strain Andrews, Subacute Bact. Endocarditis, Dr. D. Skinner	12,000-14,000	5.0
D 173	D		Strain Osakow, Subacute Bact. Endocarditis, Dr. D. Skinner	1,000-2,000	2.0
C 745	D		Meningitis, Dr. J. M. Neill	6,000-10,000	2.5
H 69D5	D		Strain Heazman, Normal Human Vagina, Dr. R. Hare	800-1,000	5.0
"	D		Strain Heazman, Normal Human Vagina, Dr. R. Hare	8,000-12,000	5.0
"	D		Strain Heazman, Normal Human Vagina, Dr. R. Hare	80,000-120,000	5.0‡
"	D		Strain Heazman, Normal Human Vagina, Dr. R. Hare	800,000-1,200,000	5.0‡

Strain	Group	Type	Source	Inoculum No. bacterial cells	O.U.* inhibiting growth
C 1	D		Cheese, Dr. O. T. Avery	1,000-2,000	5.0
"	D		" " " " "	10,000-20,000	5.0
K 129	E		Milk, Dr. J. H. Brown	7,000-10,000	0.0625
C 24A	E		Swine, lymphadenitis, Dr. H. J. Stafseth	10,000-12,000	0.0313
F 67	F		Strain Forrester II, Human Sinus Infection, Dr. E. A. Bliss	5,000-6,000	0.0625
C 41F	F		Rockefeller Hospital, Empyema, Dr. G. S. Mirick	1,000-2,000	0.0625
H 127	F		Rockefeller Hospital, Tonsil	500-600	0.0313
1RSC161	G		Rockefeller Hospital, Acute Pharyngitis, ? Scar. Fever	2,000-3,000	0.0156
4RS72	G		Rockefeller Hospital, Acute Pharyngitis	3,000-4,000	0.0156
F 78B	G	(Minute)	Normal pharynx, Dr. Helen Plummer	3,000-4,000	0.0313
C 518	G		Strain 3702, human throat, Dr. F. Schwentker	7,000-10,000	0.0078
Monk. 37	G		Monkey, Pneumonia, Dr. Thomas Francis	5,000-10,000	0.0156
F 90A	H		Strain Perryer, Normal human throat, Dr. R. Hare	6,000-7,000	0.0156
K 208	H		Rockefeller Hospital, Alveolar abscess	6,000-7,000	0.0156
D 34F	K		Normal pharynx, Dr. R. Hare	5,000-6,000	0.0625
K 130	L		Certified Milk, Dr. J. H. Brown	5,000-6,000	0.0156
D 167A	L		Strain SCH 16, Dog (Vagina), Dr. R. M. Fry	3,000-4,000	0.0313
D 168AX	M		" " 192, Dog or Fox, Dr. R. M. Fry	25-35	0.0019
"	M		" " " " " " " " " "	250-350	0.0019
"	M		" " " " " " " " " "	2,500-3,500	0.0039
"	M		" " " " " " " " " "	25,000-35,000	0.0039
D 168BX	M		" " 200 " " " " " " "	600-700	0.0039
C 559	N		*S. lactis*, Strain O. J., Milk, Phyllis M. F. Shattock	1,000-2,000	0.0625

* O.U.—Oxford Unit.

† Undet.—Undetermined.

‡ An occasional colony appeared on subculture.

TABLE II.

Results of Tests for Penicillin Inhibitor in Broth Filtrates and Acetone-Ether Extracted Cells of a Group D and *E. coli* Strain.

System 10–5 dilutions Strain C716 plus	Dilutions of penicillin									
	1:2*	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
0.3 cc broth (control)	—	—	—	—	—	—	—	+	+	+
2 mg extracted H 69D5 cells	—	—	—	—	—	—	—	+	+	+
0.3 cc H69D5 broth filtrate	—	—	—	—	—	—	—	+	+	+
2 mg extracted *E. coli* cells	+	+	+	+	+	+	+	+	+	+
2 mg extracted *E. coli* cells (heated to 56°C 30 min)	+	+	+	+	+	+	+	+	+	+
2 mg extracted *E. coli* cells (heated to 65°C 30 min)	—	—	—	—	—	—	—	+	+	+
0.3 cc *E. coli* broth filtrate	+	+	+	+	+	+	+	+	+	+
0.3 cc *E. coli* broth filtrate (heated to 65°C 30 min)	—	—	—	—	—	—	—	+	+	+

* First tube contained 2.0 Oxford Units of penicillin.

+ indicates growth of test strain, C 716.

— indicates no growth of test strain, C 716.

ported here show that penicillin in amounts varying between 0.0039 and 0.0625 Oxford units sterilized an inoculum containing 1,000 to 10,000 cells of all the strains tested belonging to serological groups A through N with the exception of group D strains which required 2.0 to 5.0 units to inhibit completely the growth of a similar sized inoculum. Group M strains were the most sensitive; group A, C, G, H, and L strains were next; and group B, E, F, K, and N strains were the least sensitive of those found susceptible to the action of penicillin in small amounts.

From our experience as well as that of others,[11,12] it appears that with the doses and methods of administration currently used in patients, concentrations of more than 1 Oxford unit per cc of blood would be difficult to maintain for an appreciable length of time. It, therefore, seems unlikely that human infections with group D streptococci that require systemic treatment would respond readily to penicillin therapy in amounts commonly used now. Possibly localized infections, where higher concentrations of penicillin could be maintained, might respond favorably to this drug. On the other hand, infections with strains of groups A, B, C, E, F, G, H, L, K, M, and N would be expected to respond favorably to penicillin since they are susceptible in concentrations that can be readily maintained in either the circulation or locally.

By the methods used, no penicillinase or inhibitor of penicillin could be demonstrated in either the extracted cells or filtrate of a broth culture of a resistant group D strain, although with similar methods an inhibitor which was destroyed or inactivated by heat (65°C for 30 min.) was demonstrated in both the cells and filtrate of a broth culture of a strain of *E. coli.* No attempts were made to further identify this inhibitor. If group D streptococci do not produce substances which destroy or inactivate penicillin, they probably will not interfere with the action of the drug on other penicillin-susceptible microörganisms when found in mixed infections.

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[11] Watson, R. F., Rothbard, S., and Swift, H. F., *J. Am. Med. Assn.*, 1944, **126**, 274.

[12] Rammelkamp, C. H. and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 425.

14701

Effect of Ether Extract of the Thymus and Pancreas on Synthesis of Acetylcholine.*

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It was found that less acetylcholine was synthesized in the presence of serum[1,2] and spinal fluid[3] from patients with myasthenia gravis than from healthy persons or patients with diseases other than myasthenia gravis. The pathological changes demonstrable at autopsy may be an enlarged thymus and lymphocytic infiltration of certain tissues. Patients with myasthenia gravis may benefit from removal or irradiation of the thymus. It was ascertained in the following that the synthesis of acetylcholine is modified by the presence of thymus extracts.

Experimental. Series I. The effect of minced thymus on the synthesis of acetylcholine *in vitro* was studied in a few experiments, but the results were inconstant. Since the various constituents of the minced thymus

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* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

[2] Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

[3] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

TABLE I.
Effect of Ether Extracts of Various Tissues on the Synthesis of Acetylcholine *in vitro.*

Substance	No. of separate extracts	No. of exp.	Amount of acetylcholine synthesized in % control*			
			Free acetylcholine In presence of frog brain and human		Total acetylcholine In presence of frog brain and human	
			spinal fluid	serum	spinal fluid	serum
			(1)	(2)	(3)	(4)
Controls†		50	100	100	100	100
Thymus	10	20	65	63	62	64
Pancreas	5	10	58	59	54	56
Lymph gland	5	10	100	97	100	106
Thyroid	5	10	113	105	112	108
Salivary gland	4	8	97	99	105	109
Lung	3	6	102	98	104	105
Subcutaneous fat	2	6	103	105	105	110

* The S.E. of the mean for each value was less than ±5%.

† The amount of acetylcholine synthesized in μg per 100 mg of frog brain in the control mixtures and the S.E. of the mean was: (1) 2.11 ±0.053; (2) 1.45 ± 0.011; (3) 3.16 ± 0.049; (4) 2.08 ± 0.029.

may have different or opposing effects on the synthesis of acetylcholine, an ether extract of the thymus was prepared by the method described by Novinski.[4] Fresh minced thymus was immersed in acetone for 6 hours, then the tissue was extracted in a continuous ether extractor for 24 hours. The tissue was discarded and the ether extract evaporated to dryness. Similar extracts were prepared from pancreas, thyroid, lymph gland, salivary gland, lung, and subcutaneous fat.

The synthesis of acetylcholine was studied by the method of Quastel, Tennenbaum, and Wheatley[5] with minor modifications.[2] Two to 5 mg dry extract were suspended in a mixture containing 100 mg minced fresh frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, 2 cc Ringer's solution, and 1 cc human serum or spinal fluid. The pH of the mixtures was adjusted to 7.4. Identical mixtures without the dry extracts served as controls. The mixtures were shaken and incubated aerobically for 4 hours. After incubation the amount of free and total acetylcholine synthesized was assayed biologically by the use of the sensitized rectus abdominis muscle of frog.

The amounts of acetylcholine synthesized in the presence of the extracts are summarized in Table I. While extracts of thymus and pancreas depressed the synthesis of acetylcholine, extracts of the other tissues did not modify it. It is not likely that the decrease found in the presence of the extracts of thymus and pancreas was due to impurities introduced during the extraction, since extracts of the other tissues did not modify the amount of acetylcholine synthesized.

The nature of the factor responsible for the decrease in the synthesis of acetylcholine is not yet known. Dr. W. H. Summerson was kind enough to ascertain the ultraviolet absorption spectrum of some extracts. The extracts did not have a maximum of absorption between 2,400 and 3,000 Å, therefore they did not contain a demonstrable amount of either nucleoproteins or most of the decomposition products of nucleoproteins.

Series II. In the following series of experiments cats and rabbits were anesthetized with a Dial-Urethane solution (Ciba).† Blood samples were collected from the femoral artery. An equal volume of Locke's solution was introduced into the femoral vein. 150 mg of the dry extract of the thymus, pancreas or lung dissolved in 1 cc of ether, or 1 cc of ether alone, were injected subcutaneously.

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[4] Novinski, W. W., *Biochem. Z.*, 1930, **226**, 415.

[5] Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Biochem. J.*, 1936, **30**, 1668.

† The Dial-Urethane solution when added to the mixtures before incubation did not modify the synthesis of acetylcholine *in vitro*.

TABLE II.
Effect of Injection of Ether Extract of Various Tissues on Properties of Serum.

Animal	Extract injected in ether	No. of exp.	Amt of acetylcholine synthesized in the presence of serum taken after injection of the extract expressed in percent of the amount of acetylcholine synthesized in the presence of serum taken before injection (= 100% base line)* Time of collection of blood (hr) following injection ½	1	2	3	4-6	7-10	22
			Free acetylcholine						
Cat	thymus	4	95	72	66	64	60	66	102
Rabbit	”	2			62		57		96
Cat	pancreas	3		74	64	66	62	71	101
”	lung	3		96	105	95	102	107	101
”	(ether)	4		99	97	100	103	104	102
Rabbit	”	2			62		55		94
			Total acetylcholine						
Cat	thymus	4	98	79	67	61	63	66	99
Rabbit	”	2			62		55		94
Cat	pancreas	3		75	60	62	59	73	97
”	lung	3		98	103	94	98	105	108
”	(ether)	4		100	96	100	102	103	100
Rabbit	”	2		101		99	100		

* The S.E. of the mean for each value was less than ± >5%.

Blood samples were collected hourly for 10 hours and again after 22 hours. The effect of the serum on the synthesis of acetylcholine was studied as above. In these experiments the incubated mixtures contained 100 mg frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, 1 cc cat serum, and 2 cc Ringer's solution.

The results are summarized in Table II. The average of the amounts of acetylcholine synthesized in the mixtures containing serum collected before injection of the extracts served as the 100% base-line. The synthesis of acetylcholine did not change significantly in the presence of serum collected during the period of 22 hours from cats injected with ether and with the ether extracts of lung. However, in the presence of serum collected over a similar period following the injection of extracts of thymus and pancreas, the synthesis of acetylcholine was decreased as much as 45%. Serum collected 22 hours after the injection no longer reduced the synthesis of acetylcholine.

Discussion. The above results indicate that ether extracts of thymus and pancreas contained some substance in the presence of which less acetylcholine was synthesized. Serum of cats injected with ether extracts of thymus and pancreas exerts a similar effect on the synthesis of acetylcholine. The serum of the injected cats was probably not a simple mixture of the extract and serum. It is probable that the composition of the serum actually was changed since samples collected from 6 to 10 hours after injection exerted a greater effect than samples collected earlier. It is possible that the factor responsible for the decrease of acetylcholine synthesis is more readily soluble in ether than in water. Our experiments, however, do not exclude the possibility that the substance is ubiquitous and the extraction by ether may have served only to separate it from many impurities, which, by themselves, had some effect upon the synthesis of acetylcholine.

Traces of the extracts of thymus and pancreas were sufficient to reduce the synthesis of acetylcholine to a degree comparable to the decrease of synthesis found in the presence of serum[1,2] and spinal fluid[3] from patients with myasthenia gravis. Therefore it is possible that a decrease of the synthesis of acetylcholine may occur if this factor be released from the thymus into the blood stream.

Summary. 1. The effects of ether extracts of thymus, pancreas, lymph gland, thyroid, salivary gland, lung, and subcutaneous fat on the synthesis of acetylcholine were investigated. 2. Extracts of thymus and of pancreas significantly decreased the synthesis of acetylcholine *in vitro* while the other extracts had no demonstrable effect on this process. 3. Less acetylcholine was synthesized in the presence of serum from animals injected with the extract of thymus and pancreas than in the presence of serum from non-injected animals or animals injected with extracts of lungs. 4. The extracts of the thymus and pancreas contained neither nucleoproteins nor their decomposition products in an amount demonstrable with ultraviolet spectrography.

14702

Studies on Heavy Metal Poisoning: I. The Use of Natural Radioactivity for Tracer Studies on Uranium.*

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Introduction. Since uranium was introduced as an experimental nephropathic agent by Leconte[1] several attempts have been made to develop a method to measure small quantities of uranium such as are encountered in biological materials in animals poisoned with this heavy metal, but none of these methods has utilized the natural radioactivity of uranium as the basis. Recent developments in the measurement of emanations from radioactive substances have made this possible and with the aid of Dr. A. E. Ruark in the Physics Department a reasonably satisfactory method has been developed.

Our immediate interest in the problem arose from studies by Donnelly and Holman[2] in which it was shown that the intravenous administration of sodium citrate protected dogs against a lethal dose of uranium nitrate. If a method could be perfected for determining small quantities of uranium, it might be possible to determine whether sodium citrate leads to increased elimination of the heavy metal by altering the solubilities of the complex uranium salts, or otherwise. Before these studies could be completed they were interrupted by one of us (W.A.D.) being called away to participate in the war effort. The results to date have indicated: (1) that the method has merit, (2) that uranium nitrate disappears rapidly from the blood stream after intravenous injection, and (3) that approximately ⅔ of the uranium can be recovered in the urine in the first 24 hours after injection.

Methods. Four adult dogs fed the regular kennel diet and allowed free access to water were used. Two of these dogs received daily intravenous injections of sodium citrate (0.33 cc of a saturated aqueous solution [approximately 230 mg] per kg) for 5 days before the injection of uranyl nitrate and for 5 additional days following the injection of the heavy metal. Each of the 4 dogs received an intravenous injection of 5.0 mg/kg of uranyl nitrate ($UO_2(NO_3)_2 \cdot 6H_2O$, Merck in 0.5% aqueous solution) accurately transferred by washing out the syringe with blood. Immediately after the injection of uranium nitrate the dogs were catheterized and placed in clean metabolism cages. All urine passed during the next 24 hours was collected. In each case the 24-hour urine output was diluted up to 1500 cc with cage washings. Blood samples were taken immediately before and at 4, 19, 34, 64, 124, and 244 minutes after injecting the heavy metal—also at the end of 24 hours. The blood samples consisted of 12 cc of venous blood (jugular vein) mixed with 2.0 cc of 1.4% sodium oxalate in a 15 cc graduated centrifuge tube and centrifuged at 3,000 r.p.m. for 35 minutes.

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* This investigation was aided by a grant from The John and Markle R. Markle Foundation.

[1] Leconte, *Gas. Med. Paris,* 1854, IX, 488, (cited by MacNider, W. deB.), *Physiol. Rev.,* 1924, **4**, 595.

[2] Donnelly, G. L., and Holman, R. L., *J. Pharm. and Exp. Therap.,* 1942, **75**, 11.

Corrections were made for this oxalate dilution in the figures recorded. Similar oxalated plasma obtained from normal dogs was used in preparing the standard dilutions from which the calibration curves were made.

Standard calibration curves were prepared by making standard dilutions of uranium nitrate covering the approximate range of dilution determined by previous trial, viz., 1:10, 1:100, 1:200, 1:400, and 1:800. The alpha-particle activity of these standard dilutions of uranium in blood plasma and in urine-blood-plasma (see below) was determined, and the uranium content of the unknown samples was determined from these calibration curves.

The sample of each specimen of standard and unknown blood plasma was 5.0 cc. In the case of the standard and unknown urine analyses, 5 cc of urine were mixed with 5 cc of oxalated blood plasma and these 10 cc of oxalated blood plasma-urine were analyzed. All of the samples were evaporated to dryness at about 110°C for 2-4 hours in flat porcelain dishes, ashed at 650-750°C for 3 hours, cooled, immersed in a liter beaker containing 700 cc of distilled water for 24 hours, the water in the beaker drawn off with a siphon, and the water in the crucible cover evaporated to dryness at 110°C. It was found that this washing for 24 hours removed some of the soluble salts which screened the activity of the uranium complex. Several analyses of the concentrated wash water showed that it never contained as much as 2% of the material washed, and most of the analyses were entirely negative.

The thin crystalline film in the bottom of the crucible cover had a hard glazed appearance and measured about 25 micra in thickness. Since this is greater than the range of the alpha particles, these films were considered as "thick" sources. The alpha particle activity was counted by an ionization chamber operating a linear amplifier and mechanical counter. The distance from the film to the entrance screen of the ionization chamber was 0.55 cm and the effective entrance area of the opening was for the alpha particles proceeding vertically 3.40 sq cm. All counts were carried on for at least one hour, and in the case of "weak sources" counting was continued for 4-5 hours.

The background or natural counting rate of 20 per hour was subtracted from the count of each specimen measured.

The root-mean-square error in the amount of uranium associated with statistical fluctuations alone is about $100/\sqrt{N}$ if expressed in percent. Here N is the total number of counts. This expression does not make allowance for fluctuations of background but is sufficiently accurate for present purposes. In actual practice this narrowed down to a probable error of about 10% and a maximum error of about 25%.

Experimental Observations. The standard calibration curves for plasma and urine are shown in Fig. 1 and 2 respectively. The points plotted are the average of duplicate determinations, and all of them fall along a straight line within the range tested. It was necessary to add protein to the urine in order to obtain these maximal reproducible results. We do not know the reason for this. Crude chemical and physical tests showed that the straight ashing of standard aqueous and urine dilutions changed the uranium to U_3O_8. Possibly the crystals that formed without the added plasma protein were too large and resulted in some screening, for the counts were much lower with this simple direct technic. Wet ashing with nitric acid did not improve the technic, but we have not exhaustively studied wet ashing methods.

Regardless of the absolute value of the method, when the same arbitrary technic was applied to standard dilutions and to unknown samples of oxalated dog plasma at varying

FIG. I

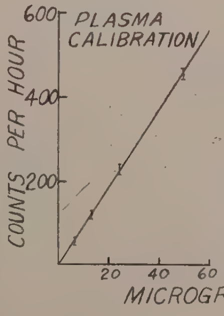

FIG. 2

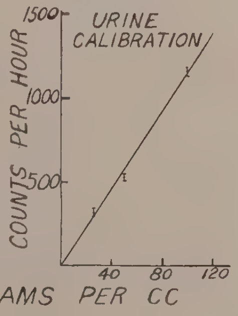

TABLE I.

Recovery of Uranyl Nitrate in Urine in 24 Hours after Intravenous Injection of 5.0 mg/kg.

Dog No.	Sex	Estimated age (yrs)	Body wt (kilos)	Total uranyl nitrate inj. (mg)	Total uranyl nitrate recovered in urine (mg)	% recovery in urine in 24 hr
39-38	M	6	18.6	93.0	72.0	77
39-42	M	5	24.3	121.5	37.5	31
43-33	M	3	18.4	92.0	81.0	88

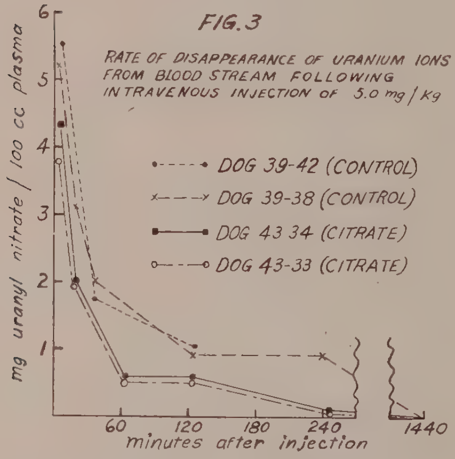
FIG. 3

RATE OF DISAPPEARANCE OF URANIUM IONS FROM BLOOD STREAM FOLLOWING INTRAVENOUS INJECTION OF 5.0 mg/Kg

intervals after the intravenous injection of a minimal lethal dose of uranium nitrate, it was found that the uranium disappeared rapidly from the blood stream. At the end of 4 hours radioactivity in the blood stream was minimal and by the end of 24 hours the count had returned to background level in all 4 dogs (Fig. 3).

It was somewhat of a surprise to us to recover so much of the uranium in the urine that was passed in the first 24 hours after the injection (Table I). This varied from 31% to 88% of the total amount injected and averaged 65% for the 3 dogs tested. Even if the maximal error of 25% occurred in the dog with the highest percentage recovery (dog 43-33) there would still be a recovery of 66% of the amount injected. Unfortunately these studies were not carried on beyond the first 24 hours and no analyses of tissues or bile have been attempted, hence we have no quantitative checks on the total amount injected.

Discussion. It is difficult to compare the results obtained by our method with those obtained by other methods, for different amounts of different preparations were administered by different routes to different species of animals. Karsner and Reimann[3] using a chemical method with potassium permanganate titration found about 20% of 50.0 mg/kg in the kidneys of cats after an unstated interval. In another series of experiments they recovered 11% and 14% of the uranium injected in dogs (0.5 mg/kg) in the gallbladder bile 8 and 11 days later. Jones and Goslin[4] using a magneto-optic method on the blood, urine, liver, spleen, and kidney of a group of rabbits sacrificed at hourly intervals from 1-24 hours following the subcutaneous injection of 0.5 mg/kg of uranyl nitrate found no uranium in the bile, but an average of 20% in the liver and 23% in the kidney. About ⅓ of the uranium was eliminated in the urine in the first 24 hours. These workers noted a rapid disappearance from the blood in the first 6 hours even though the heavy metal was administered subcutaneously. Our results compare more favorably with those of Jones and Goslin, but indicate that in dogs receiving 10 times as much uranium intravenously as did the rabbits by the subcutaneous route, the heavy metal disappears more rapidly from the blood and about twice as much of the uranium is recovered in the urine in the first 24 hours.

We believe that our method possesses certain advantages over either of the 2 methods cited above. The method might be adopted by certain industrial concerns for periodically testing the urine of those exposed to radioactive compounds.

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[3] Karsner, H. T., and Reimann, S. P., *J. Med. Res.*, 1918, **39**, 157.

[4] Jones, H. D., and Goslin, R., *Am. J. Physiol.*, 1933, **105**, 693.

Even though these studies are incomplete a word or two about the immediate problem that attracted our interest to this method, viz., the protective influence of sodium citrate against uranium injury[2] might not be out of place. The 2 dogs which did not receive citrate (39-38 and 39-42) died on the 9th and 8th days respectively while the 2 dogs which did receive citrate injections (42-33 and 43-34) are both alive 12 months after the uranium injection. Both the rate of disappearance from the blood stream and the highest percentage recovery in the urine (88%) were in the dogs that received citrate (dogs 43-33 and 43-34). This might indicate that citrate brings about a more rapid elimination of the heavy metal, but if ⅔ of the heavy metal is eliminated in the first 24 hours without citrate it is difficult to see how citrate administered as late as 67 hours after uranium can be equally effective in protecting against the heavy metal (see next paper).

Summary. 1. A method is described whereby the alpha-particle radioactivity of uranium can be utilized to follow its course in the body. 2. Four hours after the intravenous injection of a minimal lethal dose of uranium nitrate (5.0 mg/kg) practically none of the injected heavy metal can be detected in the blood plasma. 3. Within the first 24 hours after the intravenous injection, approximately ⅔ of the uranium is eliminated in the urine. 4. Similar tracer studies have been applied to dogs protected by sodium citrate injections, but no satisfactory data have been obtained to explain the manner in which sodium citrate protects against a lethal dose of uranium nitrate.

14703

Studies on Heavy Metal Poisoning: II. Sodium Citrate Therapy Effective 67 Hours After Uranium Injury.*

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Last year we reported the survival in 13 of 14 dogs (92%) that had received intravenous injections of sodium citrate for 5 days before and 5 days after a lethal dose of uranium nitrate. Twelve of 13 control dogs (without citrate) succumbed to the same dose of heavy metal (5.0 mg/kg). One week after the injection of uranium the degree of azotemia, of acidosis, of alterations in the urine, and of histological change in the kidney in the two groups of dogs was similar.[1] Apparently something happened between the 5th and 8th day that determined the fate of the dogs.

The timing of the citrate injections in these experiments, *i.e.*, before and after the administration of uranium, was related to the fact that these studies grew out of other studies in which sodium citrate was used as an anticoagulant. Because of theoretical and practical considerations we have repeated these experiments but have given the citrate injections only *after* the uranium poisoning.

Methods. The methods were the same as those employed in the previous study[1] except that the daily intravenous citrate injections (0.33 cc/kg—approximately 0.23 g—of a saturated aqueous solution of tri-sodium citrate) were started at varying intervals *after* the subcutaneous injection of uranyl nitrate ($UO_2(NO_3)_2 \cdot 6H_2O$, 0.5 mg/kg in 0.5% aqueous solution). Blood samples for NPN and CO_2 determinations and urine samples

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* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

[1] Donnelly, G. L., and Holman, R. L., *J. Pharm. and Exp. Therap.*, 1942, **75**, 11.

TABLE I.
Effect of Sodium Citrate Injections on Survival in Dogs Given a Lethal Dose of Uranium Nitrate.

Group	First citrate inj. given	Citrate inj. continued for days	No. of dogs	No. surviving	% survival
I	Simultaneously	10	5	5	100
II	(a) After 17 hr	10	5	5	100
	(b) ” 17 ”	5	6	5	83
III	” 67 ”	5	4	4	100
	Total		20	19	95

for PSP, protein, and microscopic analyses were collected on the 3rd, 7th, and 12th day after the injection of the heavy metal.

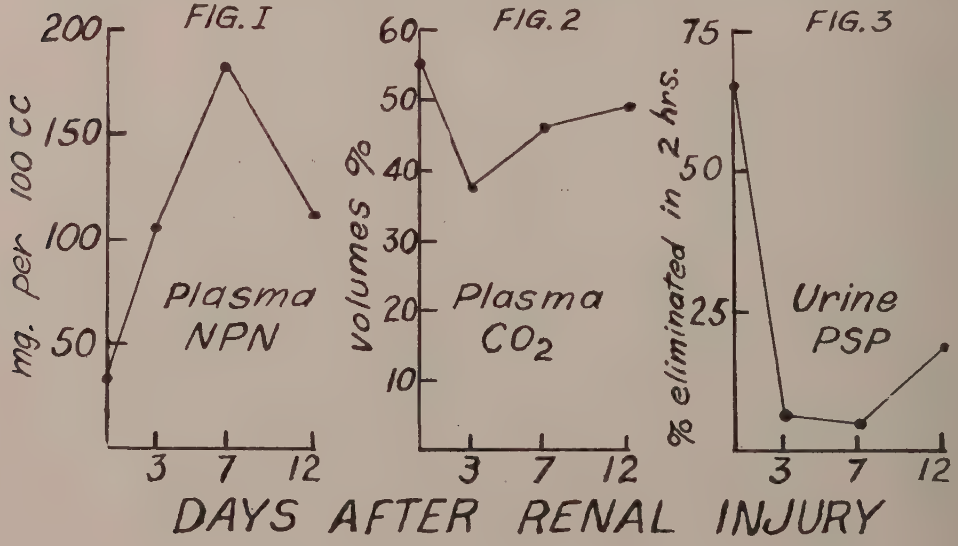
Experimental Observations. Table I gives the survival figures for the group of 20 dogs. Sodium citrate injections started as late as 67 hours after the administration of uranium and continued for only 5 days were as efficacious as 10 daily injections started immediately after the injection of the heavy metal. And the survival figure for the entire group, 95%, is the equivalent of that obtained in the previous series (with citrate injections before and after the uranium injections) in which 13 of 14 dogs (92%) survived.

In Fig. 1, 2, and 3 are presented the changes in NPN, CO_2, and PSP following the heavy metal injury. The points plotted represent the average for the entire group as obtained on the 3rd, 7th, and 12th days after the uranium injection and are practically identical with those obtained in the previous study except that the CO_2 combining power of the plasma did not fall quite as low and started returning toward normal sooner than in the previous study.

The clinical condition of the dogs remained good throughout. All reactions were averted by giving the citrate injections very slowly. No abnormalities in the clotting of blood were noted at any time. The single fatality (Group IIb) occurred on the 12th day.

Anatomical studies in this series of dogs were limited to biopsy and necropsy material

Effect of Sodium Citrate Injections on Blood Chemical and Urinary Excretion Studies in Dogs Given a Lethal Dose of Uranium Nitrate.



obtained from 3 weeks to 9 months after the renal injury. The kidneys of the dog that died on the 12th day showed considerable evidence of regeneration. By the 3rd week approximately ⅔ of the damaged tubules were lined by newly formed flattened epithelium. Anatomical material at later periods showed a progressive decrease in the amount of this "flattened epithelium," and most of the kidneys examined 9 months after the injection of uranium showed normal broad columnar epithelium lining the proximal convoluted tubules.

Discussion. The practical implications of these studies on acute uranium poisoning are obvious. We have not made any studies on chronic poisoning by uranium. The only report that we know of in which sodium citrate has been used to counteract heavy metal poisoning is that by Kety and Letonoff[2] in which sodium citrate gave beneficial results in human cases of lead poisoning. It is possible that some of the success usually attributed to plasma and indirect whole blood transfusions might be due to sodium citrate.

The theoretical considerations of these studies involve not only the method whereby sodium citrate protects against uranium ions but also the pharmacological action of the heavy metal itself.

There are several points that militate against the currently accepted view that sodium citrate exerts its protective action by preventing or combating the acidosis associated with uranium nitrate injury:

1. The alkali reserve as expressed by the CO_2 combining power of the plasma is not maintained by sodium citrate [Fig. 2 and (1, Fig. 2)].

2. The acidosis is the result of and not the cause of the renal injury.[3]

3. Sodium citrate does not prevent the curious coagulative necrosis of the proximal convoluted tubules of the kidney which classically follows uranium nitrate injury (1, Fig. 3-8).

4. If the results in the preceding paper in this series[4] are confirmed—namely, that approximately ⅔ of the uranium is eliminated in the urine in the first 24 hours—it is difficult to see how citrate administered 67 hours later can counteract the effects already produced by uranium.

Rather our data indicate that sodium citrate maintains one or more vital equilibria during the critical period (5th-8th day) when regeneration is taking place. This would indicate "replacement" or "supplemental" therapy which is directed at something more fundamental than the acid-base balance.

Possibly uranium damages a specific substance which is formed or concentrated in the cortex of the kidney. Indirect evidence pointing to a selective action by uranium is not lacking: (1) there is little or no reaction about the injection site in the subcutaneum, the obvious site of maximum concentration of uranium ions; (2) within 12 hours after subcutaneous injection there is functional evidence of renal injury (proteinuria) and at the end of 24 hours anatomical evidence of selective damage in the proximal convoluted tubules is demonstrable. Thus the lack of correlation between the concentration of uranium ions and the site of anatomic injury and between the time of maximal concentration at the site of damage (if ⅔ of the total amount injected is lost from the body in the first 24 hours) and the time of effective therapy (as late as 67 hours after the injection) could be used to support the selective affinity hypothesis.

This selective "toxic action" of uranium might logically occur by competition between uranium ions or electrons and those of another cation (perhaps a trace element) in an enzyme system with consequent inhibition of that system. The fact that uranium is hexa-valent makes this hypothesis attractive; and some of the other findings associated with uranium injury (glycosuria and increased susceptibility of pregnant animals[5]) could be put on a more logical basis if this or a similar hypothesis could be established.

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[2] Kety, S. S., and Letonoff, T. V., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 476.

[3] Karsner, H. T., Reimann, S. P., and Brooks, S. C., *J. Med. Res.*, 1918, **39**, 177.

[4] Holman, R. L., and Douglas, W. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 72.

[5] MacNider, W. deB., *J. Phar. and Exp. Therap.*, 1929, **35**, 31.

Whether or not this hypothesis holds true, we believe that our data on sodium citrate protection against uranium injury point to the maintenance of one or more vital equilibria (possibly the "citric acid cycle" of carbohydrate metabolism[6]) while the normal processes of repair are taking place, for we have no evidence that repair is accelerated or altered in any way.

Summary. Nineteen of 20 adult dogs survived a lethal dose of uranium nitrate (5.0 mg/kg) when large doses of sodium citrate (0.23 g/kg/day for 5-10 days) were injected intravenously. The citrate injections were as effective when started as late as 67 hours after the heavy metal as when started immediately after the injection of uranium nitrate.

Objections are raised to the currently accepted view that maintenance of the alkali reserve is the mechanism whereby protection is afforded, and the suggestion is offered that sodium citrate supplies ions or molecules that keep the vital "citric acid cycle" of carbohydrate metabolism going while recovery takes place.

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[6] Krebs, H. A., and Johnson, W. A., *Biochem. J.*, 1937, **31**, 645.

14704

Maternal Inheritance of Protoporphyrins of the Harderian Glands in Mice.

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Data indicating a parallelism between the amount of protoporphyrins in the Harderian glands of mice and intrinsic susceptibility to cancer of the mammary gland have been published.[1,2] Mice belonging to inbred strains with a high incidence of spontaneous mammary tumors have a very high concentration of protoporphyrins (deep red fluorescence under ultraviolet light); mice of strains with intermediate degrees of tumor susceptibility show an intermediate "pink" fluorescence; and mice of cancer-resistant strains exhibit very little or none of the fluorescence characteristic of protoporphyrins. Moreover, female mice of a cancer-susceptible strain (C_3H) have a higher concentration of protoporphyrins in their Harderian glands than males of the same strain and age.[3] *Only* female mice of a cancer-susceptible strain develop breast cancer spontaneously.

The problem of immediate interest is, can further studies on the determination of protoporphyrin concentration throw additional light (or reveal further parallelisms) on their level and the phenomena of susceptibility and resistance to spontaneous cancer? Among the most interesting (and perhaps the most important) observations in the analysis of the genetic influence on cancer susceptibility and resistance in mice is the finding that susceptibility to cancer of the breast in the F_1 individual is determined by the female parent (maternal inheritance).[4]

Chart I presents the data obtained on the red fluorescence (hence protoporphyrin concentration) in mice of the (a) C_3H strain (high or maximal protoporphyrin in the Harderian gland and high cancer susceptibility), (b) JK strain (minimal or low protoporphyrin and low cancer susceptibility), and (c) the 2 F_1 classes obtained by crossing mice of the parental stocks in both directions. The first F_1 generation was obtained by crossing a

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* This experiment has been made possible by grants from The Anna Fuller Fund and The Jane Coffin Childs Memorial Fund for Medical Research.

[1] Strong, L. C., and Figge, F. H. J., *Science*, 1941, **94**, 331.

[2] Figge, F. H. J., Strong, L. C., Strong, L. C., Jr., and Shanbrom, A., *Cancer Research*, 1942, **2**, 335.

[3] Strong, L. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 123.

[4] Little, C. C., and Staff Roscoe B. Jackson Memorial Laboratory, *Science*, 1933, **78**, 465.

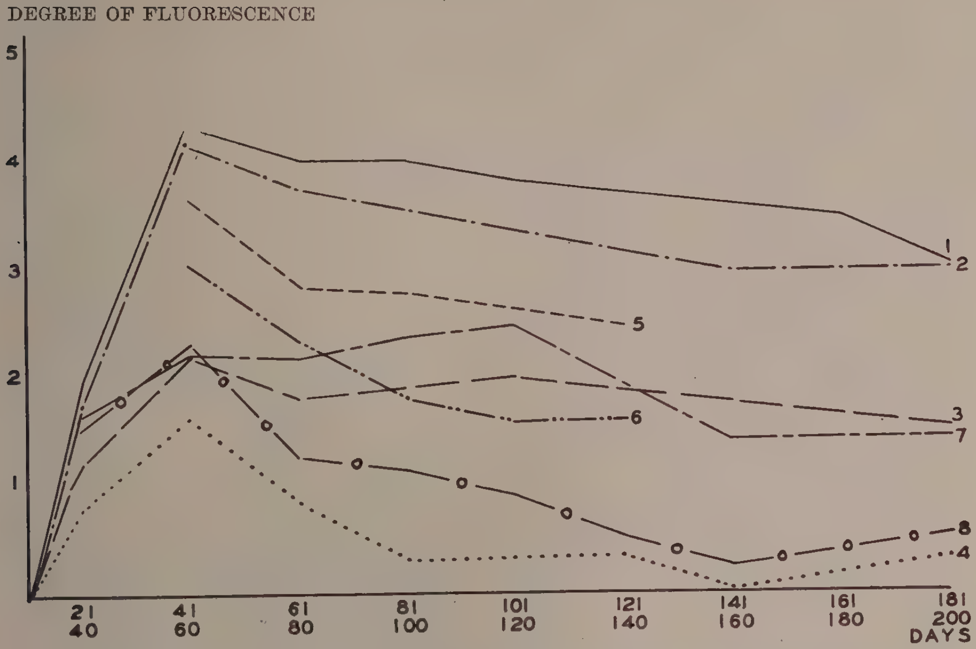

CHART 1.
This chart presents the data on the degree of red fluorescence under ultra-violet light (presence of protoporphyrins of the Harderian glands of mice). The reading of fluorescence is given on the vertical line; the age of the mice along the base line. Data on the C_3H are given on curves (1) female, and (2) males. Data for the JK mice on curves (3) female and (4) males. Data on F_1 (C_3H females × JK males) on curves (5) female, and (6) male. Similar data on F_1 (JK females × C_3H males) on curves (7) female and (8) male.

female of the C_3H strain with a male of the JK strain, and the second F_1 generation obtained by crossing a JK female with a C_3H strain male. Data obtained on the two sexes are presented separately. 1826 mice were used in this experiment—the data being divided into the various points (age) indicated in Chart I. At least 5 mice were used at each age point, thus producing a reliable curve for each of the groups.

An inspection of the chart will disclose the following facts: (a) in all classes the degree of red fluorescence rose rapidly, beginning as soon as the eyes were open and continuing through adolescence and into early sexual maturity, and then gradually declined, with advancing age, at approximately the same rate, in all cases. The main difference between the groups was the height attained by the red fluorescence before the decline set in; (b) in all cases the reading for the female was higher (approximately 25% higher, consistent with previous chemical extraction studies) than for the males of the same strains and classes;[3] (c) the reading for the F_1 generation was between the corresponding reading of the two ancestral stocks; and (d) the reading of the F_1 generation was closer to the reading for the female ancestral stock than for the male stock (maternal inheritance).

Conclusion. Thus the conclusion seems justified that another parallelism between cancer susceptibility and the presence of protoporphyrin in the Harderian gland is evident (maternal inheritance). Such a series of observations (even though they be in the nature of parallelism or correlation only) seems to be more than fortuitous. The level of protoporphyrins in the Harderian gland is probably a reflection of protoporphyrin metabolism throughout the body, and whether protoporphyrins (probably by a sensitizing action) have any effect on cancer or not must be tested further by the suitable administration of protoporphyrins to experimental animals. This experiment is in progress.

14705

Simultaneous Infection of Laboratory Animals with Murine and Classic Typhus. Immunological Results.

M. Ruiz Castaneda and Roberto Silva.
From the Typhus Laboratory of Mexico.

The intranasal inoculation of mice with murine or classic typhus produces pneumonia which may be 100% fatal provided the inoculum is rich enough in rickettsiæ. Rats similarly treated are affected only by inoculation with murine virus. However, an inoculum containing large numbers of classic rickettsiæ may kill the animals although microscopic examination of smears from the lungs shows so few organisms that it would be worthless to attempt to use such material to obtain rickettsial suspensions.

It was considered of interest to study the effect of the inoculation of mice and rats with mixtures of murine and classic viruses, hoping to find evidence of a simultaneous growth of the 2 rickettsiæ and perhaps improve the antigenic value of vaccines prepared in these rodents.

Mice inoculated intranasally with mixtures of murine and classic viruses showed generalized pneumonia which killed the animals in about 48 hours. Transfers from mouse lung to other mice were successful for 13 generations, then were lost because of secondary infection. According to experiments to be discussed in a future paper, both viruses were present in the lungs at the 10th generation. The inoculation of rats with mixtures of murine and classic typhus prepared from the lungs of mice infected separately or with the lungs of mice treated with mixtures of both viruses, produced pneumonia which killed the animals in 72 to 96 hours. Transfers from rat to rat were successful through 2 generations only because of considerable contamination by secondary invaders. The possibility of a simultaneous growth of both types of rickettsiæ was investigated as follows:

From the lungs of rats killed when very ill, we prepared saline suspensions with which guinea pigs were inoculated by intradermal route. The animals developed typical skin lesions and were bled 20 days after the inoculation for complement fixation tests.

From the lungs of rats killed 72 hours after the infection, we prepared suspensions of rickettsiæ according to a method described elsewhere.[1] The final product was a rich suspension of rickettsia bodies practically free

TABLE I.
Complement Fixation with Monovalent and Bivalent Guinea Pig Sera and Monovalent and Bivalent Rickettsial Antigens.

	Dilution of serum							
Sera	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Antigens
Classic	4	4	4	4	4	2	0	Classic
”	4	2	0	0	0	0	0	Murine
”	4	4	2	2	0	0	0	Bivalent
Murine	4	4	2	0	0	0	0	Classic
”	4	4	4	4	4	2	0	Murine
”	4	4	4	4	2	0	0	Bivalent
Bivalent	4	4	4	4	2	1	0	Classic
”	4	4	4	4	3	1	0	Murine
”	4	4	4	4	1	0	0	Bivalent

0 indicates that no complement fixation was observed.
From 1 to 4, various degrees of complement fixation.

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[1] Castaneda, M. R., *Brit. J. Exp. Path.*, 1941, **22**, 167.

TABLE II.

Monovalent and Bivalent Vaccines Prepared from the Lungs of Rats, Tested Against Classic Typhus.

Guinea pig No.	Vaccinated with 2 doses, 1 cc each	Daily temperature in degrees C after inoculation with classic typhus														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Monovalent murine diluted to 1:20	.2	.4	40.0	.9	.5	40.1	40.3	40.4	41.0	40.7	40.6	40.3	40.0	.6	.8
2		.4	.5	.7	.6	.3	40.3	40.1	.7	.4	.8	.9	40.0	40.0	.5	.7
3	” ” ” ” 1:30	.4	.8	.9	.8	40.1	40.7	40.1	40.3	40.7	40.9	40.8	40.7	40.6	40.1	.9
4		.7	.7	.9	.6	.4	.8	.9	41.0	40.6	40.7	40.9	40.8	40.3	.8	.7
5	” ” ” ” 1:40	.6	.6	.8	.9	.9	.8	.6	40.9	41.7	41.0	41.2	40.9	40.5	40.0	.9
6		.7	.5	40.0	.9	.3	.7	.9	40.2	40.1	40.0	40.3	40.2	40.1	40.0	.4
7	” ” ” ” 1:50	.3	.6	.9	.3	40.0	.8	40.4	40.9	40.8	41.1	40.5	40.3	.7	.8	.9
8		.4	.3	.8	.5	40.3	.8	40.6	41.0	41.2	40.6	40.7	40.2	.9	40.0	40.0
9	Bivalent diluted to 1:20	.4	.3	.9	.9	.0	.6	.7	40.0	.6	.7	.6	.8	.7	.6	.6
10		.4	.6	40.0	.8	.9	.4	.4	.4	40.3	.8	.8	.7	.5	.4	.5
11	” ” ” 1:30	.3	.5	40.0	.7	.4	.7	.3	40.0	.7	.7	.6	.7	.8	.3	.6
12		.6	.6	40.5	.9	.5	.6	.2	.6	40.9	40.1	40.0	.4	.5	.4	.8
13	” ” ” 1:40	.4	.4	.9	.8	.2	.4	.6	.8	40.9	40.1	40.0	.4	.5	.4	.5
14		.6	.6	.7	.7	.4	.6	.1	41.4	.8	.8	.7	.7	.8	.7	.9
15	” ” ” 1:50	.6	.4	.9	.8	.8	.2	.7	40.0	.9	.9	.7	.5	.8	.9	.6
16		.6	.3	.4	.6	40.1	.0	40.2	40.3	.9	.8	.7	.5	.6	40.0	.8
17	—	.6	.5	.8	.9	40.0	40.3	40.8	40.2	40.0	40.4	40.6	40.1	40.0	40.0	.6
18	—	.5	.6	.9	.8	40.0	40.1	40.2	40.7	40.6	40.0	40.7	40.5	40.3	40.5	.8
19	—	.6	.5	.5	.6	40.3	40.0	40.6	40.3	40.5	40.5	40.8	40.7	40.0	.6	.6

.0 to .9 are abbreviations for 39.0 to 39.9°C.
40.0°C and over are not abbreviated in order to show the intensity of the febrile reaction.

from cellular detritus. This suspension was prepared in such concentration that a 1:10 dilution corresponded to No. 2 of the McFarland scale, thus having an equal rickettsial content to our monovalent concentrated murine vaccine.

The theoretically "bivalent vaccine" was tested for specificity and potency by means of selective complement fixation tests[2] and by protection experiments in guinea pigs comparing the vaccine with a monovalent murine vaccine.

For complement fixation tests the "bivalent vaccine" was titrated with sera from guinea pigs bled after recovering from murine and classic typhus. The titration was made comparing the antigen with known monovalent murine and classic antigens prepared from the lungs of mice. Table I shows the results of tests made with monovalent and bivalent guinea pig sera and monovalent and bivalent antigens. It is apparent that the bivalent antigen acts as a mixture of murine and classic rickettsiæ.

According to previous experiments, the monovalent murine vaccine protected 3 out of 4 guinea pigs against classic typhus when the animals were vaccinated with a 1:10 dilution of the stock rickettsial suspension. For the comparative protection experiment we vaccinated guinea pigs with dilutions of the stock vaccines ranging from 1:20 to 1:50. Care was taken to have equivalent suspensions of monovalent and bivalent vaccines. Eight guinea pigs were vaccinated with monovalent vaccine and 8 with the bivalent rickettsial suspensions. Two animals were treated with each dilution, administering subcutaneously 2 doses of 1 cc each at weekly intervals. The vaccinated animals were inoculated with 0.1 g of the brain of a guinea pig infected with the "Breinl" strain of typhus, 18 days after the last dose of vaccine. Table II shows the results of this experiment, including 3 controls.

It may be seen in Table II that the temperatures of the animals vaccinated with "bivalent vaccine" show many more figures below 40°C than were found in the group of animals vaccinated with the monovalent murine vaccine. It is evident, therefore, that vaccines prepared in rats with mixtures of murine and classic viruses produce a better protection against classic typhus than the material obtained after the inoculation with murine virus alone.

The serologic and protection tests here described seem to indicate that the inoculation of mixtures of murine and classic viruses produce a mixed infection in which the classic virus seems to have an important part.

Summary. Typhus rickettsiæ of the classic type which have no tendency to produce massive infection in rats, seem to multiply more readily when associated with murine rickettsiæ. The animals inoculated by intranasal route produce "lung vaccines" of higher immunizing value against classic typhus than monovalent murine vaccines.

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[2] Plotz, H., *Science*, 1943, **97**, 20.

14706

Distribution of Alkaline Phosphatase in Kidney Following the Use of Histochemical Azo Dye Test.

MAUD L. MENTEN, JOSEPHINE JUNGE, AND MARY H. GREEN.

From the Department of Pathology and the Renziehausen Foundation, University of Pittsburgh and Children's Hospital, Pittsburgh, Penn.

The technic of a histochemical azo dye test for the demonstration of alkaline phosphatase in kidney has recently been described in detail by us.[1] The test consists of a direct coupling of calcium β-naphthol, following its release from calcium β-naphthol phosphate by renal

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[1] Menten, M. L., Junge, J., and Green, M. H., *J. Biol. Chem.*, 1944, **153**, 471.

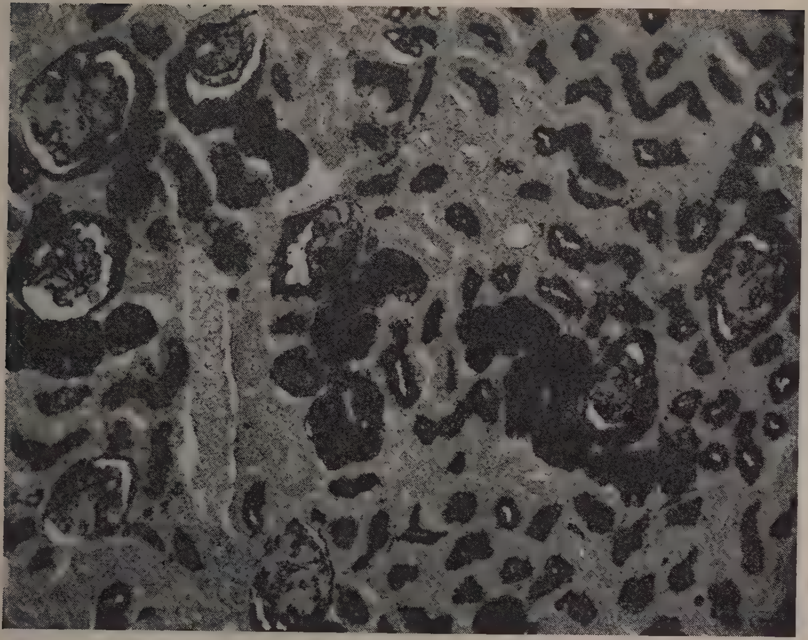

Fig. 1.

Mouse kidney: Dye deposit in glomerulus, capsular space, capsular epithelium, and the brush border and lumen of the proximal convoluted tubule. × 220.

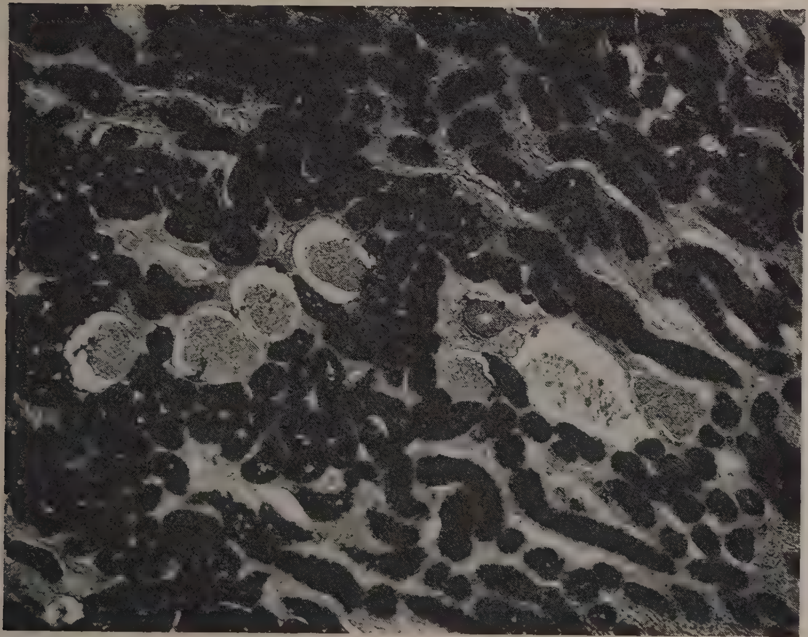

Fig. 2.

Rat kidney: Dye deposit in the brush border and lumen of the proximal convoluted tubule. A trace of dye occurs in some of the glomeruli. × 110.

phosphatase *in situ,* with a diazotized amine. This coupling forms an insoluble reddish purple dye. The loci of the precipitated dye in paraffin sections of kidney indicates the site of the phosphatase activity.

Mouse, rat, and human kidney were studied. The alkaline phosphatase was found mainly in the cortex where it occurred in greatest abundance in the proximal convoluted tubules. The medulla, except in one or two instances, was free of enzyme.

In the nephron of the adult mouse, dye deposition began in the glomerulus in moderate amount, was slight in the flattened epithelium of the upper half of Bowman's capsule and reached a high concentration in the columnar lining of the lower half of the capsule. The capsular space also contained quantities of the dye. The dye extended down in maximal amount into the first part of the proximal convoluted tubule and gradually decreased toward the lower straight segment (Fig. 1). The remainder of the nephron contained little or no dye, with the exception of one instance, a mouse, in which the precipitate was abundant in the distal convoluted tubules. Details of distribution in the different parts of the nephron were of considerable interest. The greatest amount of dye occurred in the brush border and in the adjacent lumen. The beaded line stood out like a string of small reddish purple beads. The mitochondria in the epithelium of the first part of the proximal convoluted tubule frequently contained considerable dye. In the tuft the amount varied in individual animals, but was generally in greater concentration in the epithelium than in the endothelium. The endothelium appeared as an irregular wavy ribbon beneath which the basement membrane was well demarcated as a thin homogeneous red line. The juxta-glomerular body and the afferent arteriole often showed a moderate amount of diffuse hyaline pink color. Occasionally, other small arteries showed a similar coloration throughout their walls. The macula densa, where this structure could be identified, was relatively free of dye. In some sections a black precipitate occurred in sparsely distributed nuclei which possibly may have belonged to neutrophils. The nature of the black reaction is not known.

In the rat, the dye was limited to the cortex, occurring not only in the proximal but also in the distal convoluted tubules and with a variable deposition in many of the ascending limbs of the loops of Henle (Fig. 2). It was absent in the descending limb of the loop of Henle. The dye was seen in the epithelial lining of an occasional Bowman's capsule in moderate or slight amounts around the entrance to the neck. In the glomerular tuft, absence or only an occasional ill-defined trace of color was the rule. In the proximal convoluted tubules the precipitate was in the brush border and outer part of the lumen, and in the distal tubules in the lumen close to inner border of the cells. Rarely the granules of the mitochondria in the proximal convoluted tubules revealed the presence of dye (Fig. 3). Presence of phosphatase in the wall of the arteries of the medulla, as described by Gomori,[2] has been only rarely observed by us.

In the human kidney the distribution of dye corresponded more closely to that of the mouse kidney than that of the rat. The dye was slight or absent in the glomerulus and Bowman's capsule (Fig. 4). The amount of precipitate was moderate and was most pronounced in the first part of the proximal convoluted tubule, where it appeared in the brush border and in the lumen. The beaded line was distinctly shown as a granular red line. Occasionally, faint color was seen in the other locations observed in the kidney of the rat. The delay in obtaining autopsy material, as well as pathological changes in the human kidney, may modify the concentration of renal phosphatase, and probably accounts for our failure to confirm Gomori's[2] finding of the enzyme in the ascending loop of Henle and the cortical arterioles.

In parallel sections made from the same tissue as that employed in the azo dye technic and using glycerophosphate, phenylphosphate, and calcium β-naphthol phosphate in the Gomori test we have noted similarity of enzyme activity and distribution with the three substrates, with the possible exception of the calcium β-naphthol phosphate which required the addition of magnesium to bring out the whole activity of the phosphatase. It should be noted that the concentration of this ester was small due to its low solubility. These observations indicate that there is an identical location of the enzyme with both technics

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[2] Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

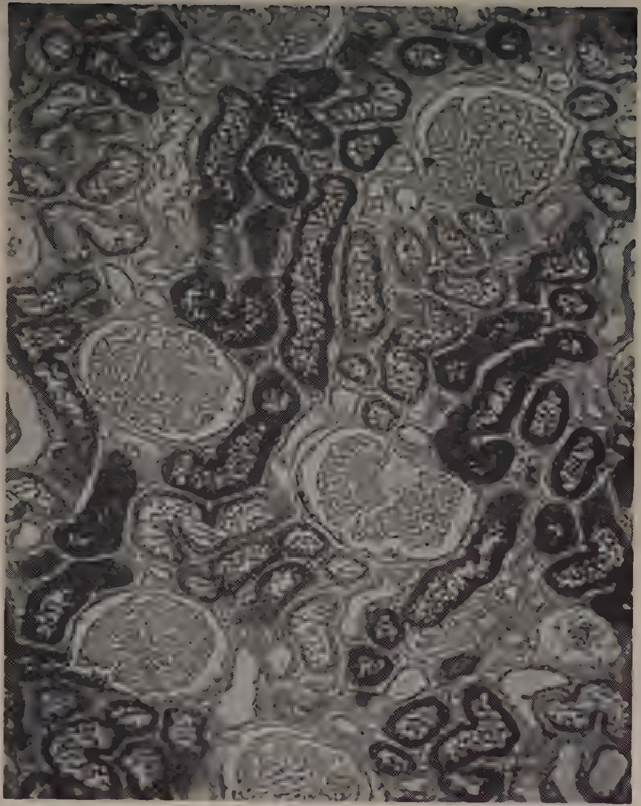

Fig. 4
Human kidney: Dye practically limited to first part of the convoluted tubule. × 110

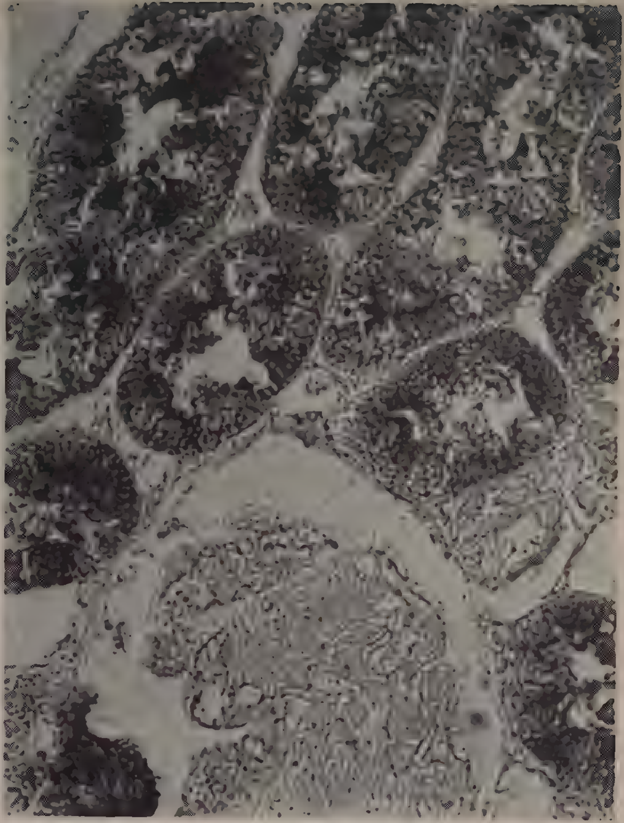

Fig 3
Rat kidney: Dye on the mitochondria of cells of the proximal convoluted tubule. × 700.

and also that a single renal monoesterase rather than multiple esterases is involved.

In general, our results confirm those reported with the Gomori[3] and the Takamatsu[4] technic. Minor differences, however, were observed, such as the presence of phosphatase in the glomerulus in contrast to its absence in this structure reported by Kabat and Furth.[5] In the study of a series of miscellaneous animals Wilmer[6] recently reported phosphatase in the glomeruli of the cat and the chicken, so that enzyme activity in this structure undoubtedly varies in different species.

It is interesting that the mouse is the only one of the 3 species studied by us in which the dye appeared in large amounts in the capsular lining which is uniquely of columnar type epithelium in this animal. The presence of the enzyme in this position, as well as in glomerulus, raises the question whether phosphatase activity and the metabolic processes with which it is associated may not be instituted at a higher structural level in the nephron of certain species than of others.

The occasional clear-cut demonstration of phosphatase in the mitochondria of the proximal convoluted tubules suggests that these cellular structures are functionally related to or are associated with the development of this enzyme in the kidney. Recently, Breedis, Flory, and Furth[7] and Wilmer[6,8] have noted that pathologic changes in the cytoplasm of the proximal convoluted tubules result in loss and disappearance of the phosphatase from their lumina. This observation gives support to the belief that the mitochondria may be the site of origin of the renal alkaline phosphatase.

Summary. The location of the alkaline phosphatase in the kidney of the rat, mouse, and human shown by the use of a histochemical azo dye test is mainly in the proximal convoluted tubule. This distribution agrees substantially with that obtained by the silver technic of other investigators. Minor differences in the enzyme distribution in the three species are described.

Thanks are due to Dr. Mortimer Cohen and Miss Ann Shiras for making the photomicrographs.

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[3] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

[4] Takamatsu, H., *Tr. Jap. Path. Soc.*, 1939, **29**, 492.

[5] Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

[6] Wilmer, H. A., *Arch. Path.*, 1944, **37**, 227.

[7] Breedis, C., Flory, C. M., and Furth, J., *Arch. Path.*, 1943, **36**, 402.

[8] Wilmer, H. A., *J. Exp. Med.*, 1943, **78**, 225.

14707

Studies in Hodgkin's Syndrome. II. A Search for Brucella in Hodgkin's Syndrome.*

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The coexistence of Brucella infection and Hodgkin's syndrome has been reported by Parsons and Poston[1] and Forbus[2] in a series of 24 cases studied by them at Duke University. The strains of Brucella which were obtained induced a mild infection when inoculated into experimental animals. There was an atypical distribution of lesions described, from which Brucella were not regularly recoverable. Specific agglutinins could not be

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[*] Studies in Hodgkin's syndrome have been aided by a grant from the Comley Research Fund.

[†] Research Fellow.

[1] Parsons, P. B., and Poston, M. A., *Southern Med. J.*, 1939, **32**, 7.

[2] Forbus, W. D., *Am. J. Path.*, 1942, **18**, 745.

TABLE I.

	Patients	Nodes	Spleens	Blood	Miscel. cultures	Postmortem study	Original cultures	Subcultures
Hodgkin's disease	35	40	2	75	24	5	725	4050
Controls	36	23	9	22	3	none studied	216	1296

demonstrated[3] in inoculated experimental animals. The suggestion was made that brucellosis may influence the course of Hodgkin's syndrome or be associated in some manner with its etiology. The testing of this hypothesis in another geographic area was the objective of the studies reported in this paper.

In a series of distribution studies Hoster[4] has recently shown that the mortality statistics of Hodgkin's disease in the United States parallel neither the mortality of human brucellosis nor the incidence of brucellosis in cattle.

Methods. At the inception of our investigation of this phase of the Hodgkin's problem, one of us visited Durham, N.C., and through the kindness of Dr. Forbus, was given the opportunity of discussing methods, observing technical details, and studying fixed tissue sections with members of the group who participated in the Hodgkin's-Brucella studies carried on in the Departments of Medicine and Pathology at Duke University.

Using the method described by Poston[5] for the isolation of Brucella from tissue and body fluids, we have studied a series of 71 patients, 35 with histologically verified Hodgkin's granuloma, and 36 with miscellaneous diseases involving the reticulo-endothelial system. The geographic distribution of patients is centered in Ohio and includes a few of the neighboring north central states. Bacteriologic studies have included cultures from nasal and throat secretions; from urine and blood; from aseptic surgical specimens of liver, spleen, lymph node, and bone marrow; from postmortem samples of spleen, lymph node, and liver; and from other miscellaneous sources. (Table I.)

The group classified under miscellaneous diseases involving the reticulo-endothelial system included monocytic leukemia, myelogenous leukemia, Cohnheim's sub-leukemic lymphatic leukemia, lymphosarcoma, metastatic carcinoma, Banti's disease, polycythemia vera, aplastic anemia, pan-splenic hematopenia, infectious mononucleosis, congenital hemolytic icterus, reticulo-endotheliosis, granuloma of unknown etiology, tuberculosis, chronic brucellosis, and fungus disease.

Results. No organisms identified as belonging to the Brucella group were isolated during the course of the present study. There were 3 patients with Hodgkin's granuloma whose brucellergin skin tests were positive after 48 hours; none, however, showed a significant agglutinin titer for Brucella and none developed an elevation of opsonocytophagic index[6] which was considered significant.

From 3 of 6 Hodgkin's patients available for intensive study during the 6-months period prior to death, large, gram negative, cocco-bacilli were obtained in pure culture. Two strains were isolated from the blood stream and one from the spleen. The morphological, serological, and cultural characteristics of the organisms could not be confused with Brucella. Selected patients were skin tested with antigens made from the 3 strains isolated, and no difference in the cutaneous reactions of individuals in the 2 groups could be observed. Using each isolated strain as a separate antigen, it has been noted that serum agglutinin titers were higher in some of the Hodgkin's granuloma patients than in those of the control group. Though further bacteriologic and immunologic studies are being carried out, the organisms isolated are considered to be of questionable etiologic significance, similar perhaps to that of Brucella in Hodgkin's disease.

Summary and Conclusions. An unsuccessful attempt has been made to isolate bacteria of the Brucella group from 35 patients with Hodgkin's disease and from 36 patients with miscellaneous diseases of the reticulo-endothelial system. Three strains of gram negative cocco-bacilli not belonging to the genus Brucella have been isolated.

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[3] Forbus, W. D., and Gunter, J. U., *Southern Med. J.*, 1941, **34**, 376.

[4] Hoster, H. A., *Ohio J. Science*, 1944, **44**, No. 6.

[5] Poston, M. A., *J. Lab. and Clin. Med.*, 1941, **26**, 1961.

[6] Foshay, L., and LeBlanc, T. J., *J. Lab. and Clin. Med.*, 1937, **22**, 1297.

14708

In Vitro Inhibition of Mycobacteria by Streptothricin.

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Aside from penicillin, streptothricin is the only antibiotic whose pharmacologic properties indicate a suitability for general use in the animal organism.[1,2] Interest in the development of an antituberculosis agent of microbiological origin led to *in vitro* tests of the efficacy of the only 2 antibiotics known which at present could possibly be of general use in animals or humans, namely, penicillin and streptothricin.

Various species of mycobacteria were totally resistant to the action of penicillin, even as

TABLE I.
Inhibition of Mycobacteria by Streptothricin.

Exp. 1. Long's medium. 6 weeks' incubation

	Units per ml			
	0	.3	1	3
M. tuberculosis (avian)	+	+	±	—

Exp. 2. Nutrient agar—pH 7.0. 4 days' incubation

	Units per ml				
	0	0.1	1.0	10.0	100
M. tuberculosis (hominis 607)	+	+	±	—	—
M. lepræ P.	+	+	—	—	—
M. smegmatis I.M.	+	+	±	—	—
M. smegmatis Tol.	+	+	±	—	—

Exp. 3. Long's medium—pH 8.0.
4 days' incubation

	Units per ml						
	0	0.1	0.2	0.3	0.4	0.6	1.4
M. tuberculosis (hominis 607)	++	+	±	—	—	—	—
M. lepræ P.	++	++	—	—	—	—	—
M. smegmatis I.M.	++	±	—	—	—	—	—
M. smegmatis Tol.	++	+	±	±	—	—	—

8 days' incubation

	Units per ml							
	0	0.1	0.2	0.3	0.4	0.6	1.0	1.4
M. tuberculosis (hominis 607)	++	++	++	++	++	+	—	—
M. lepræ P.	++	++	++	++	++	±	—	—
M. smegmatis I.M.	++	++	++	++	++	++	±	—
M. smegmatis Tol.	++	++	++	++	+	—	—	—

No change at 14 days.

+ = growth.
± = poor growth.
— = no growth.

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[1] Metzger, H. J., Waksman, S. A., and Pugh, L. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 251.

[2] Robinson, H. J., Graessle, O. E., and Smith, D. G., *Science*, 1944, **99**, 540.

TABLE II.
Bactericidal Action of Streptothricin on Mycobacteria.

	Units per ml						
	0	1.0	1.5	2.0	3.0	4.0	5.0
M. tuberculosis (var. hominis 607)	+	+	+	+	—	—	—
M. lepræ P.	+	+	+	+	+	±	±
M. smegmatis I.M.	+	+	+	+	+	—	—
M. smegmatis Tol.	+	+	+	+	+	—	—

+ = growth upon subculture on Long's agar.
± = poor growth upon subculture on Long's agar.
— = no growth upon subculture on Long's agar.

much as 1,000 Oxford units per ml. Further studies revealed that this resistance probably is due to the abundant formation of a penicillin-destroying enzyme, presumably penicillinase. Details of this are reported elsewhere.[3]

A study of the inhibitory action of streptothricin for 5 *Mycobacterium* cultures is included in Table I. Since streptothricin is stable, a single addition of the antibiotic was sufficient to maintain the desired unitage over the period of 6 to 8 weeks necessary for growth of some strains of mycobacteria. Streptothricin was particularly effective in Long's medium; this is probably the result of the alkalinity of the medium which is known to enhance the *in vitro* activity of streptothricin.[4] The inhibitory range of 0.1 to 1.0 unit of streptothricin per ml places these *Mycobacterium* cultures among the most sensitive organisms to streptothricin. *M. phlei* previously was found sensitive.[5]

A rough indication was obtained for the bactericidal action of streptothricin upon mycobacteria. Long's medium containing various quantities of streptothricin was inoculated heavily with 4 *Mycobacterium* strains. After 14 days' inhibition, portions of each culture were streaked on Long's agar to determine viability of the cells in the inoculum. Table II indicates that streptothricin was definitely bactericidal in low concentrations.

These experiments, placing the mycobacteria among the streptothricin-sensitive organisms, as well as the known therapeutic effectiveness of streptothricin against certain infections in guinea pigs and mice,[1,2] suggest the desirability for detailed *in vivo* tests of streptothricin as a possible agent in the therapy of tuberculosis.

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[3] Woodruff, H. B., and Foster, J. W., *J. Bact.*, in press.

[4] Foster, J. W., and Woodruff, H. B., *Arch. Biochem.*, 1943, **3**, 241.

[5] Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 207.

14709

Demonstration in Cotton Rats and Rabbits of a Latent Virus Related to Pneumonia Virus of Mice.*

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A previous report from this laboratory[1] described the isolation from Syrian hamsters

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* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation and in cooperation with the California State Department of Public Health.

[1] Pearson, H. E., and Eaton, M. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 677.

(*Cricetus auratus*) of a virus which produced fatal pulmonary consolidation in these animals, and which, on the basis of cross immunity tests, was shown to be antigenically related to the pneumonia virus of mice (PVM) described by Horsfall and Hahn.[2] Subsequent observations have shown that cotton rats may carry a similar agent.

A group of 6 cotton rats was inoculated intranasally with a 10% suspension of lung from an apparently normal animal of the same species. Four of the 6 rats died between 3 and 13 days later, with bright red, patchy, oedematous pulmonary consolidation. The agent causing the pneumonia was directly transmissible to mice and produced death with characteristic lung lesions in the first passage. Neutralization tests in mice using mixtures of the cotton rat lung or mouse lungs of the first passage and the serum of a rabbit immune to PVM showed specific neutralization. The cotton rats surviving inoculation with the above-mentioned normal lung had antibodies to PVM in their serum, as demonstrated by neutralization tests with a mouse-adapted strain. At various times during the past 2 years lesions similar to those described above have been seen in the lungs of cotton rats and hamsters receiving intranasal inoculations of broth or normal chick embryo tissue.

Interest in the possible occurrence of latent viruses related to PVM in other animals was aroused by the apparent production of antibodies to this virus in rabbits after the injection of plasma from persons with atypical pneumonia and of chick embryo tissues derived from serial passages started with human throat washings or plasma.[3]†

Twenty-eight rabbits from which serial bleedings were obtained had been under study from September, 1942, to December, 1943. Some of these had been inoculated subcutaneously or intraperitoneally with samples of lung tissue or sputum from persons having atypical pneumonia; some with cotton rat lung, hamster lung, or chick embryo tissue derived from serial passages initiated with lung or filtered sputum from persons having atypical pneumonia; and others with the lungs of mice infected with influenza virus. Serums from the same rabbits before inoculation and from rabbits which had been kept in the laboratory, but were inoculated only with non-infectious material, were also available.

All of these rabbit serums were tested for their ability to neutralize PVM. In these tests the mouse-adapted strain Wa, isolated in our laboratory from normal mice, was used. This strain is antigenically related to the strain used by Horsfall and his associates.[3] A 1% suspension of infected mouse lung representing 10 to 100 M.L.D. was mixed with an equal volume of rabbit serum in all tests. After incubation at 37°C for 30 minutes each of the serum-virus mixtures was inoculated intranasally under light ether anesthesia into 6 Swiss mice 4 to 6 weeks of age. The mice were observed for 12 days, at which time all survivors were sacrificed. All animals were examined for lung lesions, and the results were recorded as percentage scores[4] to indicate the degree of consolidation in each group.

In Table I details of some of the positive experiments with rabbit serums are presented. The lesion scores in mice receiving PVM plus the rabbit serum under test and in control mice receiving virus plus horse serum are given in the 2 columns to the right. Where the percentage score in the second column from the right was less than 1/3 of that in the controls (last column) definite neutralization was considered to have occurred. Similarly ratios of 1/3 to 1/2 between the lesion scores were taken as evidence of incomplete neutralization. Four rabbits were inoculated with suspensions of lung from persons who had

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[2] Horsfall, F. L., Jr., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

[3] Horsfall, F. L., Jr., Curnen, E. C., Mirick, G. S., Thomas, L., and Ziegler, J. E., *Science*, 1943, **97**, 289.

† After our studies had been completed an unpublished report of the occurrence of antibodies to PVM in normal rabbits, cotton rats, and hamsters was presented by Dr. E. C. Curnen in a round table discussion on primary atypical pneumonia at the 45th general meeting of the Society of American Bacteriologists, New York, May 3, 1944.

[4] Horsfall, F. L., Jr., *J. Exp. Med.*, 1939, **70**, 209; Eaton, M. D., *J. Immunol.*, 1940, **39**, 43.

TABLE I.
Details of Positive Neutralization Results with Rabbit Serums.

Rabbit No.	Material injected	Case	No. of injections	Date of bleeding	Interval, days*	Lesion scores in mice	
						Test serum, %	Controls, %
56	Human lung	Bl	5	11/16/42	10	100	
56	” ”	De	5	2/23/43	17	3	95
61	Mouse lung	Influenza	1	3/29	21	100	
61	Human ”	Ln	1	7/23	23	13	83
69	Nil		0	3 /9	—	93	
69	Human lung†	Lt	5	6/28	80	23	70
70	Nil		0	3 /9	—	73	
70	Human lung	De	11	1/ 8/44	88	7	70
71	Nil		0	3/12/43	—	66	
71	Chick embryo	De	8	7/23	17	10	83
66‡	Mouse lung	Influenza	1	3/26	28	90	
66	” ”	”	1	9 /8	194	3	83
68‡	Mouse lung	Influenza	1	3/26	28	100	
68	” ”	”	1	9/27	209	13	83
65	Nil	—	0	2/26	—	17	70
75	Rabbit serum ex 65	—	6	10/26	50	100	
75	” ” ” ”	—	6	12/27	112	30	83

* Interval since last inoculation.

† This suspension of lung tissue was heated to 60°C for 2 hours before injection.

‡ Rabbits 66 and 68 received 6 intramuscular injections with carbon tetrachloride in August, 1943.

pneumonia. A virus described elsewhere[5] was isolated from patient De, and presumptive evidence of the same agent was found in patient Bl. No similar agent was isolated from patients Ln and Lt, and the material from the latter patient was heated to 60°C for 2 hours before being injected into rabbit 69.

Rabbit 56 after inoculation with the suspension of lung from patient Bl failed to develop neutralizing antibodies to PVM, but both this animal and rabbit 70 had neutralizing antibodies after inoculation with the lung suspension from patient De. Rabbit 71, which received chick embryo tissues from passages initiated with the same specimen of human lung also developed antibodies to PVM. Two other rabbits inoculated with chick embryo passages of other strains of the atypical pneumonia virus had antibodies to the homologous virus, but none to PVM. Rabbits 66, 68, and 75 were under experiment during the same period as those just discussed, but did not receive inoculations of any infectious material during the period indicated. They also developed antibodies to PVM. Rabbit 65 was found to have antibodies to this agent soon after arrival at the laboratory.

A summary of these and other neutralization tests with rabbit serums is presented in Table II. It is of some interest that only 2 out of 16 normal rabbits had neutralizing antibodies to PVM on arrival at the laboratory. These animals were obtained from a breeder who also raised mice but who kept his rabbits in a separate building. The rabbits receiving cotton rat, hamster, or mouse lung also showed a relatively low incidence of neutralizing antibodies. Most of the cotton rat and hamster material was from passages initiated with sputum or lung from persons having atypical pneumonia, and many of these bleedings were obtained between September, 1942, and March, 1943, or earlier than those in Table I. The mouse lung was infected with influenza virus carried at passage intervals of 3 to 4 days. Most of the rabbits counted as normal in Table II also appear under another category where results with bleedings follow-

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[5] Eaton, M. D., Meiklejohn, G., van Herick, W., and Talbot, J. C., *Science*, 1942, **96**, 518; Eaton, M. D., Meiklejohn, G., and van Herick, W., *J. Exp. Med.*, 1944, **79**, 649; Meiklejohn, G., Eaton, M. D., and van Herick, W., submitted for publication.

TABLE II.
Summary of Results of Neutralization Tests in Mice with Serums from Rabbits Inoculated with Various Materials.

Material inoculated	No. tested	No. showing neutralization	Results*
Nil (normal)	16	2	(+) (±)
Noninfective	4	4	+ + ± (±)
Human lung and sputum	7	4	+ + + +
Cotton rat or hamster lung	7	2	± (±)
Chick embryo tissue	3	1	+
Mouse lung (influenza)	4	1	±

* + and ± represent definite and incomplete neutralization respectively. Appearance of neutralizing antibodies between first and subsequent bleedings was demonstrated except where results are in parentheses to indicate that neutralization was obtained with the earliest serum specimen available.

ing the indicated inoculations are presented.

Summary and Comment. Fourteen rabbits in a total of 28 developed neutralizing antibodies to the pneumonia virus of mice (PVM) either before or during the time they were under experiment in this laboratory. The appearance of these antibodies could not be correlated with the kind of material injected or with the development of antibodies to the atypical pneumonia virus[5] or to other agents. The incidence of antibodies to PVM was definitely higher in rabbits which had been kept in the laboratory for 2 to 10 months, and thus exposed to this agent carried by other rodents in active or latent form, than in normal rabbits newly received from an outside source or in those under experiment for a short interval of time. An agent related to PVM has been isolated from the lungs of apparently normal cotton rats, but as yet no attempt has been made to isolate a similar agent from rabbits. Human serums frequently contain neutralizing antibodies to PVM,[2] but an increase in these antibodies has not been observed. Although these results do not exclude the possibility that a virus antigenically related to PVM may be carried in the human respiratory tract, they do suggest that previous experiments[3] have failed to establish the relationship of such an agent to the virus isolated in our laboratory from persons with atypical pneumonia.[5]

14710

Influence of Riboflavin on Susceptibility of Mice to Experimental Poliomyelitis.*

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In previous studies on the relation of nutrition to resistance to infection, we have described the effect of thiamine and of pantothenic acid deficiencies on the susceptibility of mice to the poliomyelitic viruses.[1,2,3] The

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* These studies were aided by a grant from the National Foundation for Infantile Paralysis, Inc.

We are indebted to Merck and Co., Rahway, N.J., for the crystalline vitamins, and to Abbott Laboratories, North Chicago, Ill., for the halibut liver oil.

[1] Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, 1943, **45**, 85.

[2] Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Inf. Dis.*, 1944, **74**, 41.

[3] Lichstein, H. C., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 3.

TABLE I.

Series No.	Diet	Virus	Route of inoc.	Days on exp. when inoculated	No. paralyzed / No. inocul.	% paralyzed
8*	Riboflavin-Low 203	Theilers F.A.	IP	5	14/27	52
	203 + 200 γ riboflavin/100 g	0.5 ml 10% susp.		5	14/27	52
	203 + 5 mg ” ”			5	12/24	50
16*	203	Theilers F.A.	IP	14	6/22	27
	203 + 200 γ ” ”	0.5 ml of 10% susp.		14	5/20	25
	203 + 5 mg ” ”			14	5/23	22
18	203	Theilers F.A.	IP	17	9/25	36
	203 + 200 γ ” ”	0.5 ml of 20% susp.		17	16/25	64
	203 + 5 mg ” ”			17	14/27	52
18a	203	Lansing	IC	107	7/13	54
	203 + 200 γ ” ”	.03 ml of 20% susp.		107	7/10	70
25	203	Lansing	IC	23	21/33	64
	203 + maintenance riboflavin inj. every other day	0.03 ml of 2% susp.		23	23/33	69
	203 + 200 γ riboflavin/100 g			23	24/29	83
25a	203 + 30 γ ” ”	Lansing	IC	60	6/20	30
	203 + 200 γ ” ”	0.03 ml of 2% susp.		60	18/27	67
38	203	Theilers GD VII	IC	23	14/33	42
	203 + 300 γ ” ”	0.03 ml of 0.1% susp.		23	12/35	35
	203	Lansing		23	20/34	59
	203 + 300 γ ” ”	0.03 ml of 5% susp.		23	26/35	74
38a	203	Theilers GD VII	IC	46	10/13	77
	203 + 300 γ ” ”	0.03 ml of 10% susp.		46	11/14	78

IP = Intraperitoneal.
IC = Intracerebral.
* Mice from outside source; all others from our own colony.

present report is concerned with similar experiments on riboflavin.

The riboflavin-low diet (203) used throughout these studies was composed of the following parts per 100: sucrose, 73; purified-vitamin-free casein,[†] 18; mineral salts,[4] 4; corn oil, 5; thiamine, 300 μg; pyridoxine, 300 μg; nicotinic acid, 5 mg; calcium pantothenate, 2 mg; choline, 300 mg; inositol, 100 mg; para-aminobenzoic acid, 100 mg; biotin, 5 μg. Each mouse received adequate quantities of halibut liver oil by mouth twice weekly. The Swiss mice obtained from our own colony ate approximately 2-3 g per day at the beginning of the experiment; after 3 to 4 weeks the riboflavin-deficient animals still consumed about 2 g per day, but began to lose weight after 5 weeks. In contrast, thiamine-deficient mice[2] ate very little at the low point of that deficiency. Aside from loss in weight, the riboflavin-deficient mice showed thinning of the fur coat, slight bleeding about the ears, and in roughly a third of the animals, some scabs over the body. To the best of our knowledge, the animals were supplied with all necessary food substances except riboflavin, since mice on this diet plus riboflavin at a 200 μg per 100 g level, appear sleek, well nourished and in apparent good health. In the several hundred mice in our thiamine series[2] no scabs or signs of hemorrhage were observed. The thiamine-low diet and the riboflavin-low diet were identical except that the respective vitamin was omitted.

The viruses employed were Lansing strain poliomyelitis and both FA and GD VII strains of Theiler's encephalomyelitis; the FA strain was given intraperitoneally, while Lansing and GD VII strains were injected intracerebrally. Mice were inoculated when signs of riboflavin deficiency appeared in the majority of the animals, usually after 14 to 24 days on the deficient diet, so as to obtain maximum paralysis at the peak of the deficiency. Various concentrations of the viruses were used, and the resulting paralysis was directly related to the particular preparation and route of inoculation. A concise summary of the experiments is given in Table I.

[†] S. M. A. Corporation, Chagrin Falls, Ohio.

[4] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

In series 8 and 16, mice from outside sources were used and were inoculated intraperitoneally with 0.5 ml of a 10% suspension of Theiler's FA virus. The period of depletion prior to inoculation was 5 days in series 8, and 14 days in series 16. In these 2 series, no difference was noted in the percentage of paralysis in any of the groups.

In series 18, mice from our own colony were employed after 17 days of depletion; they were inoculated intraperitoneally, with 0.5 ml of a 20% suspension of Theiler's FA strain. The deficient group showed 36% paralysis (9/25), as compared to 64% (16/25) in the optimum, and 52% (14/27) in the group with marked excess vitamin intake.

The results in series 18a, which included the uninoculated control mice from series 18, indicate that age does not influence the susceptibility of riboflavin-deficient mice to Lansing strain poliomyelitis. When the initial signs of deficiency were observed in the control mice (107 days after the start of the experiment, at which time the animals were 128 days old), 0.03 ml of a 20% suspension of Lansing virus was injected intracerebrally in both deficient and optimum groups with only slight difference (16%) in the incidence of paralysis. Later, in series 25a with Lansing strain, and in series 38a with Theiler's GD VII, the age factor proved to be of no significance.

In series 25, with Lansing virus used intracerebrally, the incidence of paralysis was approximately the same for those mice on a deficient diet (64%) as for those kept on a maintenance level by intraperitoneal injections of 1-3 μg of riboflavin per day (69%). On the other hand, the difference between the deficient mice and those on an optimum level, was 19%, not marked but worthy of note, in view of results in later series. The uninoculated adult mice, control groups of this series (age 81 days) were subsequently depleted of riboflavin and then placed on a suboptimum level (25a) (30 μg per 100 g of diet with an average individual daily intake of less than 1 μg per day). The resulting incidence of paralysis in the deficient group was 30% (6/20) and 67% (18/27) in the optimum group, a difference in this instance of 37%.

Theiler's GD VII strain of encephalomyelitis virus and Lansing virus were used intracerebrally in parallel groups in series 38. The Theiler series showed no significant differences whereas the optimum and deficient groups inoculated with Lansing virus showed a 15% difference in favor of the deficient animals. Uninoculated controls of this series were subsequently injected with the Theiler's GD VII strain 46 days after the start of the original experiment (38a), but the incidence of paralysis was essentially the same both for the deficient and optimally fed groups.

Discussion. Our own experiments with thiamine and pantothenic acid[1,2,3] and those of Foster *et al.*[5] with thiamine deficiency and gross inanition,[6] showed that deficient mice tended to be more resistant to poliomyelitis than were well nourished controls.

Riboflavin deficiency results in a decreased resistance against pneumococcus infection in mice,[7] and against typhus in rats.[8] In our riboflavin experiments, no significant differences have been observed with the FA and GD VII encephalomyelitis viruses. With the Lansing strain poliomyelitis virus, the differences are slight and in one series only (25a), statistically significant.‡ The trend, however, is in the same direction in all 4 experiments and therefore merits consideration. It should also be noted that the riboflavin and the pantothenic acid-deficient mice, unlike the thiamine-deficient animals, continue to eat well so that reduced caloric intake can hardly be the important explanation of the observed differences in resistance. Furthermore, in pantothenic acid-deficient mice a definite increased resistance to Theiler's virus was observed, with no change in susceptibility to Lansing virus under identical conditions. In our riboflavin series, on the other hand it is the Lansing strain which shows the differences between the optimum and the deficient groups while the Theiler's viruses demonstrate no consistent changes. May not these points indicate definite differences in the metabolism of the several viruses?

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[5] Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *Science*, 1943, **97**, 207.

[6] Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **79**, 221.

[7] Wooley, G., and Sebrell, W. H., *U. S. P. H. R.*, 1942, **57**, 149.

[8] Pinkerton, H., and Bessey, O. A., *Science*, 1939, **89**, 371.

‡ We are indebted to Professor J. H. Torrie for the statistical study.

Summary. Mice fed an adequate diet, save for various levels of riboflavin, showed no consistent differences to infection with Theiler's encephalomyelitis viruses. In four similar series (totaling 234 mice) injected with Lansing strain poliomyelitis virus, the differences were slight but the greater resistance in each series was in the deficient group.

14711

A Rickettsia-like Organism Recovered from Guinea Pigs.*

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In the summer of 1942 there appeared an unusual febrile disease at Fort Bragg, North Carolina. This mild disease was called "pretibial fever" and was an apparently new clinical entity, as described by Daniels and Grennan.[1] The disease was characterized by 3 to 5 days of fever, splenomegaly, a maculopapular eruption typically limited to the legs, and rapid return to normal health. All of the cases were quartered in the same limited area of the post, a fact of probable epidemiological significance. An army commission which studied the 1942 epidemic was unable to establish the etiology or mode of transmission of the disease. In the summer of 1943, there reappeared a small outbreak of a clinically identical disease in which the cases came from the same area of the reservation as those of the preceding year. A rickettsia-like organism was recovered from 3 of 5 guinea pigs injected with blood from one of these latter patients. Attempts have been made to show that this agent was concerned in the etiology of the disease, but it is not possible at the present time to be sure of its origin. The purpose of this paper is to record the known characteristics of the organism in the hope that future developments may clarify its significance.

Method of Isolation. Defibrinated blood was drawn from the last case of pretibial fever appearing in the 1943 outbreak and was injected intraperitoneally into 5 guinea pigs in 4 to 6 ml amounts. After incubation periods varying from 3 to 5 days, 3 of these guinea pigs became febrile. Three independent passage lines were established by the intraperitoneal transfer of pooled brain and spleen on the third or fourth day of fever and the same organism was recovered from each. After a few transfers, the agent became lethal for guinea pigs; a titration of infected spleen from the fourth passage showed deaths through the 10^{-3} dilution of a 10% emulsion.

The Organism. Poorly staining, gram-negative rods were seen in impression smears of guinea pig tissue or of egg yolk sac. When stained by the Machiavello method,[2] the material was shown to contain numerous bright red, somewhat pleomorphic, small bacillary forms, tending to be paired and often clumped

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† At present First Lieutenant, M.C., A.U.S.

‡ Fellow in the Medical Sciences of the National Research Council.

[1] Daniels, W. B., and Grennan, H. A., *J. A. M. A.*, 1943, **122**, 361.

[2] *Virus and Rickettsial Diseases,* Harvard University Press, 1940, p. 896.

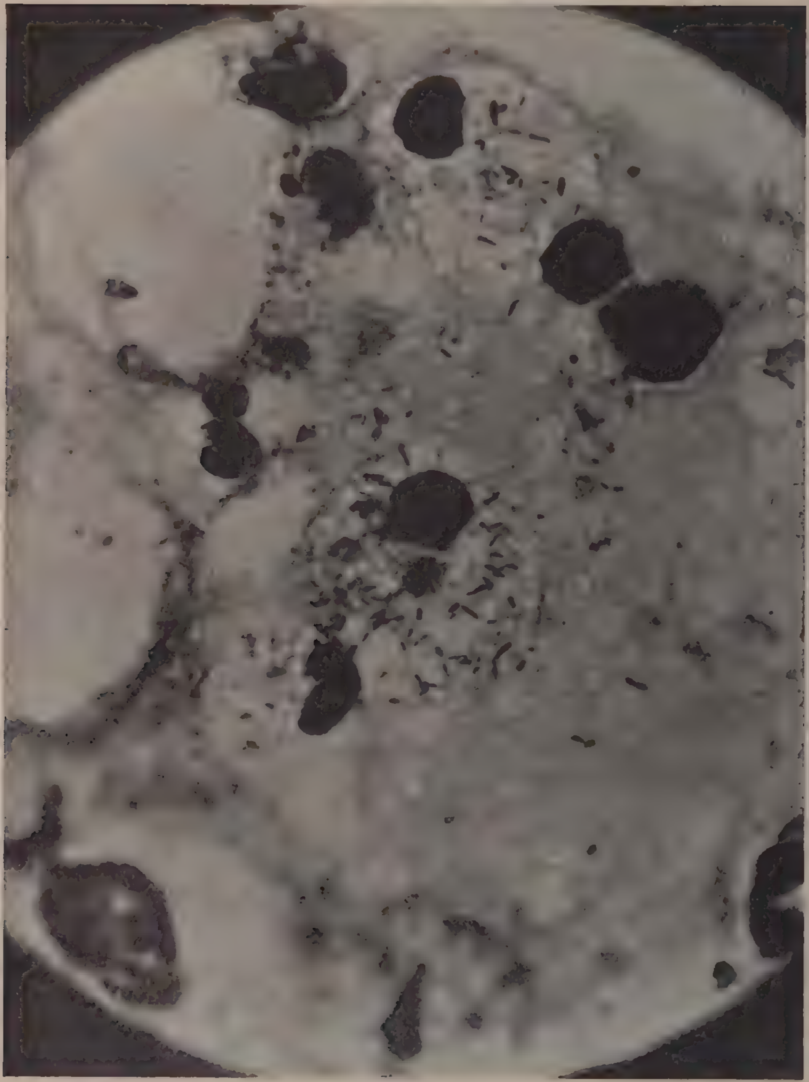

FIG. 1.
Impression smear of guinea pig peritoneal exudate stained by the Machiavello method. Magnification × *ca.* 1500.

together in bundles (Fig. 1). They were larger than *P. tularensis* and the known rickettsiæ. In contrast to the latter, these organisms were easily seen in guinea pig tissue. They were located chiefly in the cytoplasm of the cells in impression smears of the spleen, though in smears of the peritoneal exudate masses of them appeared to be extracellular. They stained purple with Giemsa's stain and were not acid fast.

Unsuccessful attempts were made to grow this organism on a variety of artificial media under both aerobic and anaerobic conditions. In addition to the common bacteriological media containing blood, special media for *P. tularensis, Leptospira, Brucella, B. pleuro-*

pneumoniæ, and *Mycobacteria* were employed without visible growth. Degenerated organisms were still visible in smears after 1 or 2 weeks on slants seeded with yolk sac material, but there was no apparent growth upon transfer to fresh media. Variable growth of the organism occurred, however, when infected guinea pig tissue was injected into the yolk sac of fertile 7-day hens' eggs. There seemed to be a predilection for the yolk sac, since eggs injected by the allantoic sac route showed organisms on smear of yolk sac tissue only. The agent was lethal for the chick embryo, which usually died on the third or fourth day even when a 10^{-6} dilution of the original 10% emulsion was employed as the inoculum. Eleven- to 13-day embryos tended to survive a little longer. After death of the embryo, the organisms became difficult to stain and many stained blue by the Machiavello method.

The pathogenicity of the organism was readily preserved for at least 2 months in the frozen state. Fever was still produced in guinea pigs when a 10^{-6} dilution in 10% serum-saline was incubated for 1 hour at 37°C and then at 4°C overnight.

Disease in the Guinea Pig. Routine passage of guinea pig spleen or peritoneal exudate was carried out by the intraperitoneal route, usually on the third or fourth day of fever. Passage of blood on the second day of fever resulted in a slight febrile response and solid immunity to reinoculation, indicating the presence of the agent in the blood stream. Guinea pigs weighing 250 g or more have appeared almost uniformly susceptible but there have been non-reactors, particularly young animals. The incubation period has varied from 1 to 4 days, depending on the strength of the inoculum. A more or less sustained febrile course has ensued, ranging from 104° to 106°F when early morning recordings were made. The animals became inactive and the fur ruffled; loss of weight occurred but no localizing symptoms developed. There were no neurological signs nor evidences of scrotal reaction. When injections were made intraperitoneally in 0.5 ml quantities, death after 4 to 7 days of fever was the rule through the 10^{-3} dilution of the inoculum. Recovery usually occurred after a week or more of fever for the 10^{-4} to 10^{-6} dilutions; 10^{-7} was the highest dilution producing fever in the injected animal. When virulent material was injected by the subcutaneous route, an extensive area of local induration gradually appeared in 4 or 5 days and the animals ran a febrile course.

Pathology. The pathology of the acute infection with this organism in guinea pigs after intraperitoneal inoculation was characterized by the presence of exudative peritonitis. There was slight redness of the subcutaneous tissue, together with variable but slight enlargement of the peripheral lymph nodes. No definite scrotal reaction was ever present, externally or internally. On opening the peritoneal cavity, from 1 to 5 ml of a thick whitish exudate was found, with an adherent fibrinous reaction over the liver and spleen. The spleen was 3 or 4 times normal size and the surface was finely granular. The adrenals appeared congested but the rest of the abdominal organs were grossly normal. The lungs occasionally showed areas of bronchopneumonia in which the organism could be seen on smear. In one instance, the cerebral meninges were injected and ill-defined collections of small round cells were seen in histological slides of the cerebral cortex. Animals which died or were sacrificed after a subacute course showed only splenic enlargement, without peritoneal exudate.

Microscopic sections of the spleen and subcutaneous tissue showed an inflammatory reaction in which mononuclear cells predominated in the acute stage of the disease. There was much necrosis in the later stages of the disease. The peritoneal exudate contained moderate numbers of polymorphonuclear leukocytes and small numbers of mononuclear cells. The organisms were easily seen in impression smears, often in large numbers, when stained by the Machiavello technic. As mentioned above, they were chiefly intracytoplasmic in the spleen and were mostly extracellular in the peritoneal exudate.

Host Range. It was not possible to transmit the infection to laboratory animals other than guinea pigs unless overwhelming doses were employed. White rats, cotton rats, hamsters, rabbits, rhesus monkeys, and different strains of mice were tried. The injection of concentrated suspensions of heavily infected yolk sac by the intraperitoneal or intravenous routes resulted in the death of young mice in 2 to 3 days. When the inoculum was diluted slightly, however, the result was asymptomatic survival. A few mice injected with concentrated suspensions developed purpuric areas about the ears and feet before they died, 2 to 3 days after injection. Unsuccessful attempts were made to adapt the agent to mice by the serial passage of liver and spleen at 5- to 7-day intervals.

Immunity. When recovered guinea pigs, including animals with no evidence of disease other than slight febrile reactions, were challenged with fatal doses of passage material, they were solidly immune. Such animals occasionally exhibited an abrupt rise in temperature within 24 hours; the fever usually disappeared in about 48 hours.

Since the organism resembled rickettsia in some respects, cross-protection tests in guinea pigs were set up with the standard strains of epidemic and endemic typhus, Rocky Mountain spotted fever, and tsutsugamushi fever. No cross immunity was shown to be present with any of these known rickettsial strains. There was no immunological relationship to Q fever by the complement-fixation test. Agglutinins for Proteus OX2, OX19, and OXK were not demonstrable in the serum of rabbits at 5, 8, and 10 days after injection with large doses of yolk sac infected with the organism. Sera from guinea pigs immune to the agent were negative in the agglutination test with *B. tularense* and *Br. abortus.*

Infected yolk sac was found to be a satisfactory complement-fixing antigen. Using the technic[3] of 1-hour fixation at 37°C in the presence of 2 full units of complement, complement-fixing antibody levels ranging from 1:16 to 1:64 (initial serum dilutions) were found in serum specimens from many of the recovered guinea pigs at about 3 to 4 weeks. These titers had fallen to less than 1:4 at 8 weeks. Complement-fixation tests were done with sera from the 7 cases of pretibial fever, including the patient from whose blood the primary isolations were made. These tests failed to show any significant rise in antibodies in the convalescent sera.

In addition, agglutination tests were performed. The organisms in infected yolk sac were centrifuged and the clarified resuspended sediment was found to be a satisfactory antigen. With the sera from two hyperimmunized guinea pigs, agglutination titers of 1:32 and 1:128 were obtained. The human sera showed no significant increases in antibody in convalescent specimens by this method.

Using guinea pigs, tests designed to detect the presence of neutralizing antibodies in both the sera of hyperimmunized guinea pigs and of cases of pretibial fever were carried out. Both infected yolk sac and guinea pig spleen were used as sources of the agent. Different methods of incubation of the serum-antigen mixtures were tried. There was no noticeable improvement in neutralization when the time of incubation was increased from 2 hours at room temperature to 37°C for 1 hour, followed by 4°C overnight. Dilutions of the infective material ranging from 10^{-3} to 10^{-6} were used. No protection was demonstrated by the convalescent human sera, but so inconclusive were the results with the immune guinea pig sera that it was felt that no reliance should be placed on these tests.

Attempts to Isolate the Organism from Stock Guinea Pigs. Three serial passages were carried out with guinea pigs from the same lot of animals which were used in the original isolations. The first passage was initiated with blood from a normal individual. There was no febrile response in any of the 6 animals and the organism was not isolated from their tissues. Both of the third passage guinea pigs proved resistant to challenge with the agent, although no complement-fixing antibodies to it were present in their serum prior to challenge. These results suggested the possibility of latency of the organisms in the stock. It should be recorded, however, that their young age (175 g, 195 g) raised the question of non-specific resistance, since non-reactors have often been noted in animals of this size. The sera from 10 normal stock guinea pigs bled 4 months after the original isolation contained no complement-fixing antibodies to the agent, though it was not known whether they came from the same source as the original animals.

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[3] Bengston, I. A., *Pub. Health Rep.*, 1944, **59**, 402.

Discussion. In these experiments a rickettsia-like organism was isolated from guinea pigs injected with blood from a patient with pretibial fever, a recently described disease of unknown etiology. It was not possible to demonstrate the presence of significant titers of complement fixing or agglutinating antibodies to this agent in the human sera. The technics employed were satisfactory for detecting these antibodies in the sera of recovered guinea pigs. Neutralization tests in guinea pigs were unsatisfactory, as is the case with most rickettsial diseases. In view of these negative findings, the origin of the agent is in doubt. Unfortunately, all of the human blood was used up at the time of the original inoculations, and no additional cases of pretibial fever appeared during the 1943 season. It is hoped that more information as to the source of the agent can be obtained in the future.

A search of the literature has failed to show a guinea pig disease similar to the one described. Mochkovski[4] reported the occurrence of a chronic rickettsiosis in the mononuclear cells of guinea pig blood. His paper describes the morphology of the organism and does not refer to the biological characteristics of the agent.

Summary and Conclusions. An agent pathogenic for guinea pigs has been isolated. The organisms appeared as small pleomorphic bacillary forms, staining red by the Machiavello method and gram negative. They were larger than the known rickettsia, as well as *P. tularensis* and *Brucella*, and were immunologically distinct from them. They grew fairly well in the yolk sac of the fertile hen's egg but were not cultivated on artificial media. The host range was limited to the guinea pig except when enormous doses were given.

After a short incubation period, peritonitis was produced in the guinea pig, for which the agent was highly virulent, and a specific immunity resulted in recovered animals. The inflammatory reaction in the tissues was characterized chiefly by the presence of mononuclear cells. The organism appeared predominantly intracytoplasmic in impression smears of the spleen.

The origin of the organism is in doubt. It has not been possible to identify it with any known group of pathogenic agents, but at present it may be termed "rickettsia-like" for descriptive purposes.

Most of the immunological procedures and much of the animal work was done at the National Institute of Health, United States Public Health Service, Bethesda, Maryland, through the courtesy of the Director.

The author wishes to express his appreciation for the assistance and advice so fully extended by the various members of the Commission on Acute Respiratory Diseases and the technical staff.

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[4] Mochkovski, C., *C. R. Soc. de biol.*, 1937, **126**, 379.

14712

Alleviation by Raw Liver of Anorexia Produced by Sulfapyridine.

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The administration of sulfapyridine to dogs in blacktongue did not interfere with the alleviation of symptoms by nicotinic acid but did prevent weight restoration.[1,2] Raw liver,

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[1] West, R., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 369.

[2] Schaeffer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 679.

TABLE I.
Effect of Liver on Sulfapyridine Induced Anorexia in Rats.

Sulfapyridine, %	Liver	Initial rat wt, g	Total wt gain, g	Basal diet consumption, g per day
—	—	204	51	15.2
1	—	201	17	10.5
1.5	—	196	2	9.3
2	—	197	4	8.9
—	5% powder	189	49	13.4
1.5	” ”	206	0.6	6.4
—	5% concentrate	202	56	14.7
1.5	” ”	193	5.4	7.9
—	2 g raw per day	221	36.5	16.7
1.5	” ” ” ” ”	215	13.2	12.9
—	5 ” ” ” ”	237	35	15.4
1.5	” ” ” ” ”	230	18	11.2
—	10 ” ” ” ”	178	48	11.5
1.5	” ” ” ” ”	180	38	9.1
—	Raw liver *ad lib.*, no basal diet	97	29	—
1.5	” ” ” ” ” ” ”	101	30	—

however, counteracted the sulfapyridine effect. The weight loss in blacktongue is due to anorexia[3] and it seemed possible that the effects observed with sulfapyridine were not due to a specific interference with nicotinic acid metabolism by the drug but rather to a sulfapyridine-induced anorexia. In the present work it has been found that raw liver counteracts sulfapyridine-induced anorexia in normal rats and dogs.

Experimental. Rats. The basal ration used in these experiments was as follows: casein, 25; sucrose, 57; cottonseed oil, 10; cod liver oil, 3; salt mixture,[4] 5. Each 4 kg contained thiamine, 12 mg; riboflavin, 20 mg; calcium pantothenate, 120 mg; pyridoxine, 12 mg. When sulfapyridine or whole dry liver powder was included in the diet an equivalent amount of sucrose was omitted. Raw pork liver was liquefied in a Waring blendor and sulfapyridine incorporated in the brei to provide the same concentration (dry weight) used in the basal diet. The raw liver and basal diet were fed in separate vessels and, except when the liver was offered *ad libitum,* the liver was always completely consumed. The results are presented in Table I and are expressed as the mean values obtained with groups of 12 male rats (Vanderbilt strain) each for 14 days.

Sulfapyridine fed as 1.5% of the diet produced a satisfactory decrement in appetite and growth rate. It has been demonstrated[5] that such growth failure is due largely to the relative anorexia. Whole liver powder* and a concentrated liver extract† were without effect in this connection. The addition of raw liver to sulfapyridine-containing diets induced some increased consumption of the basal diet and definitely increased growth. Finally sulfapyridine was fed with raw liver alone and then appeared to be without effect although growth on raw liver as the sole foodstuff was inferior to that on the basal diet.

Dogs. Four adult male mongrels were offered a diet consisting of yellow corn meal, 80; casein, 8; cottonseed oil, 10; cod liver oil, 1; and $CaCO_3$, 1. To this was added 2 volumes of water and the whole was cooked for one hour. Twice weekly each dog was given a

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[3] Handler, P., and Dann, W. J., *J. Biol. Chem.*, 1942, **145**, 145.

[4] Drummond, J. C., and Watson, A. F., *Analyst*, 1922, **47**, 235.

[5] Harris, J. S., and Kohn, H. I., *J. Pharm. Exp. Ther.*, 1943, **78**, 56.

* Wilson and Company, Chicago, Ill.

† Lederle Laboratories, Inc., Pearl River, N.Y.

TABLE II.
Effect of Liver on Sulfapyridine Induced Anorexia in Dogs.

		Basal ration Period 1		Sulfapyridine Period 2		SP and liver Period 3		Liver only Period 4	
Dog	Initial wt, kg	Food intake, g/day	Final wt, kg	Food intake, g/day	Final wt, kg	Food intake, g/day	Final wt, kg	Food intake, g/day	Final wt, kg
A	11.3	1100	10.7	400	9.9	950	10.4	1150	11.6
B	8.8	1150	9.4	750	8.6	1000	9.8	1050	9.9
C	10.5	950	10.6	550	10.0	1100	11.2	1100	11.8
D	9.7	1200	10.2	900	9.3	1050	9.9	1400	11.4
Mean	10.1	1100	10.2	650	9.5	1025	10.3	1150	11.2

capsule containing thiamine, 3 mg; riboflavin, 3 mg; nicotinic acid, 10 mg; pyridoxine, 3 mg; calcium pantothenate, 20 mg; and choline chloride, 1.0 g. After 10 days on the basal diet sulfapyridine administration was started. The drug was given in tablets 3 times daily in sufficient quantity to produce a fasting morning blood level of 3-5 mg %. Ten days later each dog was offered 100 g of raw liver daily and sulfapyridine administration continued. During the fourth 10-day period the drug was withheld but the raw liver feeding continued. The results are given in Table II.

Sulfapyridine administration produced an average decrease of 58% in the food consumption of these dogs. The raw liver was consumed well and, in addition, considerably alleviated the anorexia. In a series of similar experiments, with the same animals, sulfapyridine failed to affect appetite when a stock ration containing cooked horse meat was employed.

From these data it is apparent that oral administration of sulfapyridine produces anorexia in rats and dogs. When raw liver was then added to the diet of rats a definite improvement in their growth rate was found although their appetites were not so markedly restored. However, raw liver added to the diets of dogs almost completely prevented the anorexia. The failure of blacktongue dogs to gain weight when treated with sulfapyridine and nicotinic acid[1,2] can probably be explained by the secondary anorexia produced by the sulfapyridine, since the same phenomena have now been observed with normal dogs. The possibility still remains that the *modus operandi* of sulfapyridine in this regard does involve nicotinic acid metabolism as this mechanism is still obscure. Nor is there as yet a satisfactory explanation of the effects of raw liver. It is possible that liver increases the appetite and general well being of these animals by a mechanism quite independent of that which is disturbed by sulfapyridine.

Summary. Sulfapyridine produced anorexia and consequent growth failure and weight loss in normal rats and dogs. This was counteracted by the inclusion of raw liver in the diet, but not by dry liver powder or concentrated liver extract. The failure of nicotinic acid to induce weight restoration in sulfapyridine-treated blacktongue dogs while raw liver does stimulate weight gains may perhaps be explained on the same basis.

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A Simplified Procedure for Inducing Chronic Renal Hypertension in the Mammal.*

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Recent interest in experimental hypertension makes a simple procedure for inducing this condition in laboratory animals highly desirable. The present paper describes a method which, in our experience, is simpler in technic and gives a higher incidence of chronic hypertension than those previously used. We have applied it with equal success to the mouse, rat, dog, and rabbit, and it is apparently applicable to any of the mammalian forms commonly used in the laboratory.

The 3 principal methods for inducing chronic renal hypertension in present use are based on the original procedures of (1) Chanutin and Ferris,[1] which involved partial ablation of the kidneys and was applicable only to the rat; (2) that of Goldblatt,[2] which had as its basis constriction of the renal artery, and (3) that of Page,[3] involving the application of a cellophane or silk capsule to the kidney. Other methods based on the use of toxic agents (metal salts, sera or other nephrotoxins), operations on the nervous system, ureteral ligation, irradiation of the kidneys by X-rays, etc., although of theoretical interest, are unsuitable for maintaining a colony of hypertensive animals.

In our earlier work on the rat, we utilized the procedure of Chanutin and Ferris.[1] Only a minority of the animals subjected to this procedure develop chronic hypertension, since many die of renal insufficiency when the operation is of sufficient severity to induce hypertension or, if the operation is not severe enough, fail to develop an elevation in blood pressure. It is difficult in all of the abovementioned methods to avoid a fatal ischemia of the kidney while inducing enough interference with normal renal function to cause the animal to develop chronic hypertension.

The method of Goldblatt *et al.*,[2] although of greatest importance in advancing our recent knowledge, is not readily applicable to smaller mammals and is much more difficult to apply to the dog—for which it was primarily intended—than the application of a silk, collodion, or cotton capsule to the kidney. The latter method suffers from the disadvantage that infection is relatively frequent and when applied to the rat, the incidence of hypertension is not satisfactorily great.[5] Although the application of these foreign bodies to the surface of the kidney sometimes evokes an inflammatory invasion of the cortex,[3,6] we have observed that hypertension develops in the absence of such a perinephric reaction.[7] It occurred to us, therefore, that the observed hypertension was caused by compression of the renal parenchyma without necessitating the formation of a fibrous capsule around this organ. This led to the adoption of a procedure which during the past 2 years has been found to be more satisfactory than those previously available.

The method has been used successfully in the mouse, rat, rabbit, and dog and consists essentially in passing a cotton thread or tape

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[6] Friedman, B., Jarman, J., and Klemperer, P., *Am J. Med. Sci.*, 1941, **202**, 20.

[7] Grollman, A., and Williams, J. R., *Am. J. Med. Sci.*, 1942, **204**, 73.

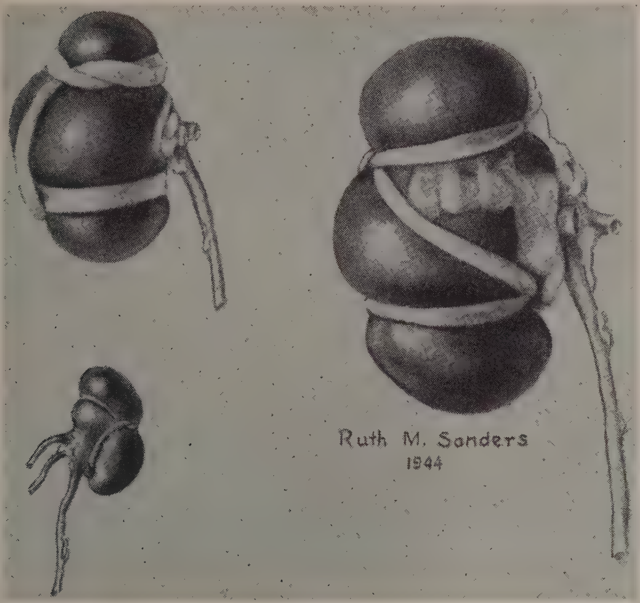

FIG. 1.
The method of compressing the kidney. In the upper left corner is the compressed kidney of a rabbit, below it, that of a rat, and at the right, that of a dog. Note the distortion of the kidney and the relation of the ligature to the hilus and poles of the kidney. The ureter, renal artery and vein are shown emerging from the hilus.

over the pole and body of the kidney and drawing it taut, as shown in Fig. 1.

After exposing the kidney by the usual lumbar incision the adrenal is pushed away from its attachments to the kidney. The latter is now brought through the incision. In the case of the rat, the renal capsule must be removed by gentle traction with the finger. Unless this is done the thread tends to slip. In the case of the dog, the tape (as shown in Fig. 1) is passed through the connective tissue which is adherent to the under surface of the kidney and is thus held firmly in position. Care is taken to avoid the ureter and blood vessels emerging from the hilus. In the case of the rat, cotton thread (No. 8 gauge) is used; for the dog or rabbit, umbilical tape has been found satisfactory since this does not cut into the renal tissues when tightened sufficiently to deform the normal ellipsoidal shape of the kidney.

The subsequent operations to be performed after compressing one kidney depend upon the type of hypertension desired and the rapidity with which it is to develop. As previously reported for the rat and rabbit, some animals (about 10 to 20%) develop a moderate degree of hypertension following the compression of a single kidney, leaving the other intact. This may require 3 to 6 months, however. If the second kidney of such animals as fail to develop hypertension in this time, be now compressed, about half the animals will be found to ultimately develop hypertension. Such animals as fail to develop the desirable rise in pressure following another interval of several months are then subjected to a unilateral nephrectomy. By following this procedure it is possible to obtain ultimately a high percentage of animals with hypertension which is relatively stable and does not rise rapidly as is the case when a more intensive form of surgical procedure is used. Animals prepared in this way are more suitable for assays and other studies in which a constant pressure is desirable over a long period of time.

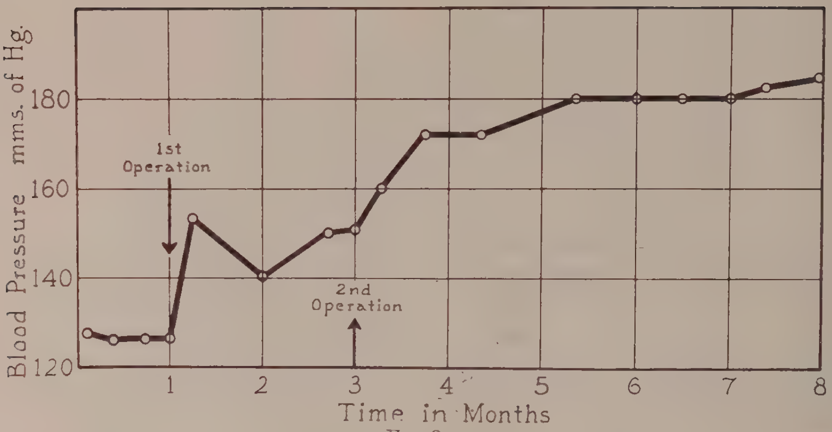

FIG. 2.

The blood pressure of a dog following compression of the kidneys. At ↓ the right kidney was compressed; at ↑, the left was similarly treated. The mean pressures as determined by direct arterial puncture and measured on a mercury manometer are given as ordinates.

If a rapidly developing hypertension is desired, the second kidney may be compressed or removed about 10 days after the first operation. Many animals thus treated will develop a rapidly progressive malignant hypertension.

In Fig. 2 is shown a typical response of the blood pressure of a dog to the bilateral compression of the kidneys. One year following the first operation, the blood pressure had gradually risen to 200 mm Hg., at which level it remained during the next 8 months during which the animal was under observation.

Summary. A relatively simple and easily applicable procedure is described for inducing chronic hypertension in the mouse, rat, rabbit, dog, or other laboratory mammal. The method in our experience results in a higher percentage of chronically hypertensive animals, is simpler in application than the procedures involving constriction of the renal artery or the application of silk or cellophane to the kidney, and is less apt to result in an interference with the renal excretory function or the development of a rapidly fatal malignant form of hypertension.

14714

Salmonella loma-linda: A New Type Isolated from Meningitis.*

P. R. Edwards.

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Salmonella loma-linda was isolated by Dr. T. F. Judefind of the College of Medical Evangelists, Loma Linda, California, from the spinal fluid of a baby that died of meningitis. The clinical and pathological aspects of the infection will be described by Dr. Judefind and his associates who recognized the organism as an unusual Salmonella type. The organism was a motile bacillus which possessed the usual cultural and biochemical

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* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

TABLE I.
Characteristics of Phase 2 of *S. loma-linda* and of *S. durban.*

	Serums			
	S. abortus equi (e,n,x . . .)		*S. glostrup* Phase 2 (e,n,z_{15} . . .)	
Antigens	Absorbed by *S. loma-linda* phase 2	Absorbed by *S. durban* phase 2	Absorbed by *S. loma-linda* phase 2	Absorbed by *S. durban* phase 2
S. loma-linda, phase 2	<100	500	<100	<100
S. durban, phase 2	200	<100	500	<100
S. abortus equi (e,n,x . . .)	500	500	500	<100
S. glostrup, phase 2 (e,n,z_{15} . . .)	200	<100	500	<100
S. abortus bovis, phase 2 (e,n,x . . .)	<100	200	<100	<100

attributes of the Salmonella group. Acid and gas were produced from glucose, arabinose, xylose, rhamnose, maltose, trehalose, mannitol, and sorbitol. No fermentation of lactose, sucrose, dulcitol, inositol, or salicin was observed. Citrate and mucate were utilized, but dextro-tartrate, levo-tartrate and meso-tartrate were not attacked. The bacillus produced hydrogen sulfide but did not form indol nor liquefy gelatin.

On serological examination the organism proved to be diphasic and its antigens were represented by the formula IX, XII:a-e,n,x . . . This is the formula attributed to *S. durban* by Henning, Rhodes, and Gordon-Johnstone.[1] However, a transplant of the original culture of *S. durban* received from Dr. Henning was found by the writer to have the formula IX,XII:a-e,n,z_{15} . . . Kauffmann[2] also found that *S. durban* was represented by the latter formula. Since *S. loma-linda* and *S. durban* were so closely related, a comparative study of two types was undertaken. The following differences were observed: Whereas *S. loma-linda* was unable or utilize either *d*- or *l*-tartrate, both were readily attacked by *S. durban.* While *S. loma-linda* did not attack dulcitol during 30 days incubation, *S. durban* produced acid and gas from the substance in 24 hours. Both cultures completely removed agglutinins from *S. gallinarum* serum and therefore have somatic antigens IX, XII. Phase 1 of both organisms was flocculated in high dilution by serum derived from *S. paratyphi* A and is represented by the symbol a. However the 2 phases are not identical since *S. loma-linda* effected a complete removal of H agglutinins from the serum while *S. durban* did not. The failure of *S. durban* to remove all H. agglutinins from *S. paratyphi* A serum was noted by Henning, Rhodes, and Gordon-Johnstone.

Examination of phase 2 of the 2 cultures revealed differences of diagnostic value. Both were flocculated by e,n,x . . . and e,n,z_{15} . . . serums. When tested with single factor x and z_{15} serums *S. loma-linda* was flocculated readily by x serum but was unaffected by z_{15} serum. On the contrary *S. durban* did not react with x serum but was agglutinated by z_{15} serum. The results of absorption tests with the 2 phases are given in Table I. It is quite apparent that phase 2 of *S. durban* is synonymous with phase 2 of *S. glostrup* and contains the antigens e,n,z_{15} . . . While phase 2 of *S. loma-linda* is not identical with that of *S. abortus equi,* it resembles that of *S. abortus bovis.* Kauffmann[3] stated that phase 2 of *S. abortus bovis* was identical with phase 2 of *S. abortus equi* but Edwards and Bruner[4] demonstrated that distinct differences existed in the e,n,x . . . phases and that phase 2 of *S. abortus bovis* lacked certain antigens found in *S. abortus equi.* The antigens of phase 2 of *S. loma-linda* are e,n,x . . .

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[1] Henning, M. W., Rhodes, F. W., and Gordon-Johnstone, J., *Onderstepoort J. Vet. Sci. and An. Ind.,* 1941, **16**, 103.

[2] Kauffmann, F., *Acta. path. et microbiol. Scand.,* 1942, **19**, 523.

[3] Kauffmann, F., *Acta. path. et microbiol. Scand.,* 1940, **17**, 1.

[4] Edwards, P. R., and Bruner, D. W., *J. Inf. Dis.,* 1941, **69**, 220.

Summary. A new Salmonella type, *S. lomalinda,* was described. The culture was represented by the antigenic formula IX,XII: a-e,n,x . . . This formula was erroneously attributed to *S. durban,* which in reality possesses antigens IX,XII:a-e,n,z_{15} . . . The two types presented biochemical as well as antigenic differences.

14715

Tuberculostatic Action of Phenothiazine and Derivatives.*

B. L. FREEDLANDER. (Introduced by S. C. Freed.)

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Phenothiazine has been proposed as an insecticide, fungicide, anthelmintic, and urinary antiseptic.[1,2] Many phenothiazine derivatives, such as methylene blue, Lauth's violet, phenothiazone, and thionol form oxidation-reduction systems. Phenothiazine, after absorption in the body, is excreted partly in the oxidized forms of phenothiazone and thionol, as well as in their respective leuko forms, with which they form oxidation-reduction systems.[3] Exposure of phenothiazine to air and light results in oxidation to phenothiazone and possibly to thionol, in which forms they become more active as fungicides. In the following experiments this interesting group of compounds was tested for bacteriostatic effect on the growth of tubercle bacilli *in vitro.*

I. The bacteriostatic action of phenothiazine was tested on the highly virulent strain of tubercle bacilli, H37Rv. Proskauer and Beck liquid synthetic asparagin media, pH 7.2, was used. Phenothiazine was dissolved in propylene glycol to make a 1% solution. Subsequent dilutions were made directly into the media. Appearance of a pellicle at the end of 3 weeks was taken as the criterion of growth. The highest dilution at which bacteriostatic action occurred was 1:1,000,000.

II. Phenothiazine and 15 derivatives were tested for tuberculostatic action on an avirulent Novy strain of tubercle bacilli. This culture normally produces a heavy surface growth within 4 days. Most of the chemical compounds were dissolved in propylene glycol. Methylene blue and Lauth's violet were dissolved in distilled water. The compounds and the results are listed in Table I. The parent compound, phenothiazine, again inhibited growth in concentration of 1:1 million. Phenothiazine sulfate also showed a high bacteriostatic effect in concentration of 1:200,000. The oxidized derivatives, phenothiazone and thionol (Nos. 3 and 4), showed a moderate degree of inhibition, in dilutions of 1:100,000 and 1:80,000 respectively. The x-hydroxyphenothiazine, apparently in the oxidized form in solution, gave results comparable with its isomer, phenothiazone. It is of interest that these oxidized forms are less effective than phenothiazine, as the reverse is the case in their inhibiting action on certain fungi. This suggests that either the oxidized forms are less readily absorbed by the tubercle bacilli, or the interference with the metabolism of the tubercle bacilli is of a different type than that of its fungicidal action. The oxidized forms are less soluble in lipids than phenothiazine; this may decrease the ability of these compounds to penetrate the tubercle bacilli in sufficient concentration.

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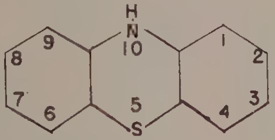
* The present report is part of a cooperative investigation on tuberculosis and has been supported by funds provided by the Committee on Medical Research of the National Tuberculosis Association, the California Tuberculosis Association, and the Rose Lampert Graff Foundation.

[1] DeEds, F., Stockton, A. B., and Thomas, J. O., *J. Pharm. and Exp. Therap.,* 1939, **65**, 353.

[2] Prelim. Report, Council of Pharm. and Chem., *J. A. M. A.,* 1940, **115**, 1721.

[3] DeEds, F., Wilson, R. H., and Thomas, J. O., *J. Pharm. and Exp. Therap., J. A. M. A.,* 1940, **114**, 2095.

TABLE I.
Tuberculostatic Action.

No.	Compound	Concentration, 1 part in:
1	Phenothiazine  H N 10 1 2 3 4 5 S 6 7 8 9	1,000,000
2	Phenothiazine Sulfate	200,000
3	Phenothiazone (3-oxyphenothiazine)	100,000
4	Thionol (3-oxy,7-hydroxyphenothiazine)	80,000
5	x-Hydroxyphenothiazine	80,000
6	Methylene Blue (3,7-tetramethyldiaminophenothiazine)	$<$10,000
7	Lauth's Violet (3,7-diaminophenothiazine)	20,000
8	N-Benzoylphenothiazine	$<$10,000
9	N-Methylphenothiazine	$<$10,000
10	5,10-Dimethyl Phenothiazonium,5-methosulfate	10,000
11	Benzo(a)phenothiazine	$<$10,000
12	Benzo(c)phenothiazine	10,000
13	x-Cyclohexylphenothiazine	$<$10,000
14	N-Nitrosophenothiazine	20,000
15	Chlorinated Phenothiazine*	$<$10,000
16	Dodecahydrophenothiazine	$<$10,000

Most of the compounds were obtained through the courtesy of the Dow Chemical Co., Midland, Mich. Phenothiazone and thionol were kindly provided by Dr. F. DeEds, Bureau of Agricultural Chemistry and Engineering, U. S. Department of Agriculture, Albany, Calif.

* One or more chlorine atoms.

The thiazine dyes, methylene blue, and Lauth's violet, showed relatively poor inhibition, as did also the remainder of the compounds. Substitution of the hydrogen attached to the nitrogen of phenothiazine by methyl, benzoyl, or nitroso groups destroyed the bacteriostatic effect of phenothiazine. In order to be effective it may be necessary that this hydrogen remain unsubstituted, to permit the compound to form oxidation-reduction systems with its leuko form.

All the compounds were tested at pH of 6.4 and 7.7. No significant differences in the tuberculostatic action of any of the compounds was noted. Phenothiazine, phenothiazone, and thionol were tested in the presence of 20% horse serum. Phenothiazine showed inhibition in concentration of 1:40,000; phenothiazone and thionol in concentration of 1:20,000. Experiments are in progress to determine the therapeutic effect of these compounds in animals.

Summary. Phenothiazine inhibited the growth of tubercle bacilli *in vitro* in high dilution. In the presence of serum the bacteriostatic effect was diminished, but was still significant. Oxidized forms of phenothiazine showed moderate inhibition of growth. Other derivatives of phenothiazine exhibited low tuberculostatic action.

14716

The Inhibition of the Succinoxidase System Using Cysteine and Cystine. II. Nature of Inhibiting Substance.*

STANLEY R. AMES AND C. A. ELVEHJEM.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

In a previous study,[1] we investigated the inhibition of the succinoxidase system using cysteine and cystine. At that time we were concerned mainly with the factors influencing the extent of inhibition and it was demonstrated that the concentration of 4-carbon dicarboxylic acids in the tissue homogenate was a determining factor. Although cysteine was added to the enzyme preparations, no inhibition occurred until it was oxidized. The nature of the inhibiting substance was therefore postulated to be cystine, but this was not definitely ascertained.

The experiments of Hopkins and Morgan[2] showed that incubation of a succinoxidase preparation with cystine would cause a loss in activity which could be entirely restored by similar incubation with cysteine. Their conclusions[2,3] were that the oxidized form inhibited and the reduced form restored the activity.

Potter and DuBois[4] discussed the inactivation caused by cysteine and cystine, and reported that both these compounds inhibited the enzyme when they were added to the final reaction mixture which contained cytochrome *c*, in the presence of which sulfhydryl is readily oxidized. However, they reported inhibitions of 51% for cystine and 55% for cysteine at the same inhibitor molarity of M/3000. In view of the fact that the effective concentration of the cysteine when oxidized is only half as great, these values are surprisingly similar. In addition we have shown[1] that in order to obtain reproducible results certain additional factors must be standardized and, therefore, the nature of the inhibiting substance merits reinvestigation.

In this investigation the inhibition caused by the cysteine-cystine system is shown to be due solely to cystine although it cannot be stated whether the dimer or the free radical is the active state.

Methods. White mice of an inbred Swiss strain were used and, after weaning, were maintained on stock ration† and water *ad libitum* plus occasional greens.

A kidney homogenate was prepared by decapitating the mice and allowing the blood to drain for a short time. The tissue was extirpated, rinsed with distilled water, and immediately packed in finely cracked ice. After cooling, bits of fat and connective tissue were removed, the kidney was blotted between moistened filter papers and rapidly weighed. A homogenate of the tissue was prepared by the technic of Potter and Elvehjem[5] in ice-cold redistilled water using a pre-chilled tube and pestle. This homogenate was immediately pipetted into the reaction flasks to which all other reactants had been previously added.

A conventional Warburg apparatus at 37°C was used in all experimental and analytical work.

The activity of succinic dehydrogenase was determined by the method of Potter[6] as modified by Potter and Schneider.[7] A summary

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* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studied were aided by a grant from the Rockefeller Foundation.

[1] Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, in press.

[2] Hopkins, F. G., and Morgan, E. S., *Biochem. J.*, London, 1938, **32**, 611.

[3] Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, London, 1939, **32**, 1829.

[4] Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, 1943, **26**, 391.

† B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, N.Y.

[5] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[6] Potter, V. R., *J. Biol. Chem.*, 1941, **141**, 775.

[7] Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, 1942, **142**, 543.

of the components of the final reaction mixture is as follows: 0.4 ml of 0.25 M phosphate buffer (pH 7.4), 0.1 ml of 4 × 10^{-4} M cytochrome *c*, 0.3 ml of 0.5 M sodium succinate (pH 7.4), 0.1 ml of 0.012 M $CaCl_2$, 0.1 ml of 0.012 M $AlCl_3$, the desired amount of homogenate, solution of inhibitor, and redistilled water to make 3.0 ml. The gas phase was air and 0.2 ml of 10% KOH and a small strip of filter paper were placed in the center well to absorb CO_2. The pH of this final reaction mixture was 7.4 as determined electrometrically.

In order to secure reproducible results, the *incubation time, i.e.,* the time the homogenate is incubated at 37°C before the inhibitor is added, and the *contact time, i.e.,* the time the inhibitor is in contact with the enzyme before the substrate is added, were carefully standardized. The numerical inhibitions recorded are valid only for a similar preparation under the identical conditions.

Eastman Kodak Company cysteine hydrochloride was used after being recrystallized from constant boiling hydrochloric acid. Solutions were freshly prepared in redistilled water and neutralized to pH 7.4 just before addition to the reaction flask.

The cystine used was isolated from hair in these laboratories. A suggestion was made in the paper of Hopkins and Morgan[2] that cystine could be brought into solution by means of a small amount of sodium phosphate buffer. In accordance with this information, it was found that cystine could be recrystallized from a sodium phosphate solution. One-half g of cystine was recrystallized in good yield from 120 ml of 0.04 M sodium phosphate buffer of pH 7.4. A stock solution was prepared in 0.05 M sodium phosphate buffer which contained 0.72 mg of cystine per ml of solution. This was supersaturated at 25°C but was stable for 12 hours or more before precipitation occurred.

Experimental. The nature of the cysteine-cystine system must be considered in some detail to see if it is possible to ascertain the nature of the specific inhibiting substance. Previous investigations have not disclosed the fate of the cysteine when it is added to the reaction mixture under consideration. It has been shown by Mathews and Walker[8] that when cysteine is oxidized in a solution of pH about 7.4, cystine is formed. When copper was added to catalyze the reaction, 100% of the theoretical oxygen uptake was achieved assuming the reaction, cysteine → cystine.

It was noted that in the reaction mixture, the addition of cysteine immediately produced a rapid oxygen uptake, but the rate of uptake soon decreased and in 20 min. had ceased entirely. Both cytochrome *c* and the cytochrome oxidase-reductase systems, added as tissue homogenate, were necessary components of the system which oxidized the cysteine. Comparison of the E'_o values at a pH of about 7 for the couples concerned,

$$\begin{array}{ccccc} \text{cystine} & & \text{cytochrome } c^{+++} & & 1/2\ O_2 \\ \upharpoonleft\!\downharpoonright & \rightleftharpoons & \upharpoonleft\!\downharpoonright & \rightleftharpoons & \upharpoonleft\!\downharpoonright \\ \text{cysteine} & & \text{cytochrome } c^{++} & & H_2O \\ -0.15\text{ v.}^{\ddagger 9} & & +0.26\text{ v.}^{10} & & +0.81\text{ v.}^{11} \end{array} \qquad (1)$$

indicated that if the above mechanism were correct, the amount of cysteine at equilibrium would be extremely small. However, in the reaction mixture, whenever cysteine was oxidized by cytochrome *c*, only about 70% of the theoretical oxygen uptake was observed (Fig. 1). Thus it would appear that there were sufficient hydrogen acceptors in the tissue homogenate to account for 30% of the theoretical oxygen uptake. This postulate may be correct since the uptake decreased from 80 to 60% with a tenfold decrease in cysteine concentration, when the same amount of tissue and cytochrome *c* were present initially. When the oxygen uptake had achieved its maximum value (about 70%), no amount of further incubation would increase the uptake, indicating that no cysteine remained to be oxidized by molecular oxygen through the cytochrome *c* system.

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[8] Mathews, A. P., and Walker, Sidney, *J. Biol. Chem.*, 1909, **6**, 21.

‡ Extrapolated to pH 7 from the E_o value given by Ryklan and Schmidt.[9]

[9] Ryklan, L. R., and Schmidt, C. L. A., *Univ. Calif. Pub. Physiol.*, 1944, **8**, 257.

[10] Stotz, E., Sidwell, A. E., and Hogness, T. R., *J. Biol. Chem.*, 1938, **124**, 11.

[11] University of Wisconsin Biochemists, *Respiratory Enzymes*, Burgess Publishing Company, 1939, page 179.

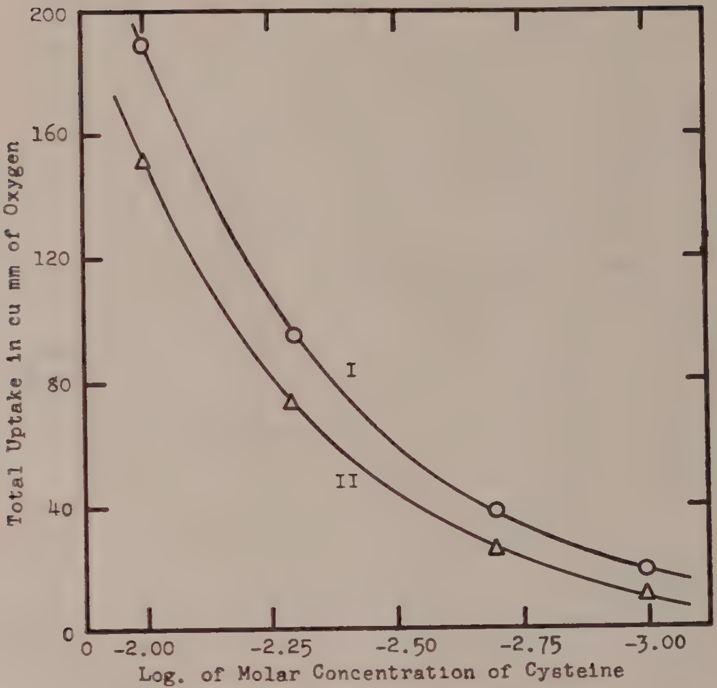

FIG. 1.

Oxidation of cysteine by cytochrome *c* and tissue homogenate. Curve I (O) represents the theoretical uptake which has been corrected to 37°C. Curve II (△) represents the uptake achieved experimentally. Test system used as described in text except that succinate was omitted. Cysteine was added from sidearm after equilibration. Five-hundredths ml of a 10% homogenate of mouse kidney was added. Concentration of cytochrome *c* was 1.3×10^{-5} M. When either the tissue concentration was reduced by half or the cytochrome *c* doubled, or both, the total uptake remained the same.

TABLE I.
Comparative Inhibitions of Cysteine and Cystine.

Exp.	Description	Q_{O_2}	% inhibition
1	Succinate control, no inhibitor present	190	
2	M/1000 cysteine, cytochrome *c* present initially	125	34
3	M/1000 cysteine, cytochrome *c* added 70 minutes after zero time	170	10
4	M/2000 cystine, cytochrome *c* present initially	113	40
5	M/2000 cystine, cytochrome *c* added 70 minutes after zero time	124	35

Test system used as described in text. Two-tenths ml of a 2% homogenate of mouse kidney was added. The incubation time was 40 min and the contact time was 30 min.

Potter[12] has shown on the basis of spectrophotometric data that in a system where no cytochrome oxidase was present, cytochrome *c* was not 100% reduced by cysteine, even with a tenfold excess. The reaction was slow and never went to 100% of the reduced form of cytochrome *c* while any cysteine was present. This perhaps indicates some discrepancy in Eq. 1, either in the E'_0 values or in the mechanism itself.

In a previous investigation,[1] we showed that cysteine alone did not inhibit the enzyme, but only after being oxidized by the addition of cytochrome *c*. It remained to show whether cysteine or some other oxidation product of cysteine was the specific inhibiting substance.

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[12] Potter, V. R., private communication.

The experiments outlined in Table I were set up to compare the degrees of inhibition of cystine and cysteine in equivalent concentrations, assuming that cysteine is oxidized to the dimer. In one pair, cytochrome *c* was added with the succinate after a 40-minute incubation and a 30-minute contact period. Cysteine without cytochrome *c* being present showed relatively no inhibition. Cysteine with cytochrome *c* present inhibited the enzyme to the extent of 34%. The oxidized dimer at half the monomer concentration and with cytochrome *c* present showed the maximum possible inhibition of 40% at M/2000 concentration (under the specific conditions noted). But in the absence of cytochrome *c* the cystine that was reduced was not reoxidized and the inhibition dropped to 35%. Cysteine with cytochrome *c* and cystine without cytochrome *c* were both lower than the maximum obtainable since the former had a time lag in the oxidation of the cysteine; the latter, nothing to reoxidize the reduced cystine. The results showed that the inhibiting substance in the cysteine-cystine system was cystine, but whether as the dimer or the free radical cannot be stated.

Summary. Cystine, without differentiating between the dimer and the free radical, inhibits the action of the succinoxidase system under the given conditions used in these experiments.

14717

On the Origin of the Fibers in the Pyramid of the Primate Brain.*

Fred. A. Mettler.

From the Department of Neurology, College of Physicians and Surgeons, Columbia University.

That the pyramid (and corticospinal system) contains fibers which do not originate from the precentral gyrus has long been known. Monakow long ago pointed out that the distribution of the pyramidal cells of the cat was more extensive than the homologous region in the primate and published a figure[1] (his 34) showing a very extensive frontal and parietal origin for the human pyramidal system. The present author observed[2], "We have seen in our parietal material that a limited number of cerebrospinal fibers arise from that part of the parietal cortex immediately adjacent to the central fissure. This is by no means a novel observation and is correlated with the occasional finding of a few Betz cells in this region." The fact that corticospinal fibers arise from a location more extensive than the precentral gyrus was subsequently verified by other workers.[3] Few persons at that time questioned the belief that the corticospinal fiber arises from anything but the Betz cell and the extensive origin of the pyramidal system was regarded as due to a widespread distribution of these cells.

The Hoffs, using the degenerated bouton terminaux technic, conjectured that corticospinal fibers arise from cortex other than type 4, and Kennard, using the Marchi method, believed she was able to establish that such fibers arise from area 6. The present writer has not been able to substantiate such an origin for the system and Levin and Bradford,[4] using the cell degeneration technic, gave it as their opinion that few if any such fibers arise

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* Aided by the Wm. J. Matheson Commission.

[1] Monakow, C. v., *Die Lokalisation im Grosshirn*, Wiesbaden, 1914, *vide* S. 183 *et seq.*

[2] Mettler, Fred A., *J. Comp. Neurol.*, 1935, **61**, 536.

[3] Levin, P. M., *J. Comp. Neurol.*, 1936, **63**, 369.

[4] Levin, P. M., and Bradford, K., *J. Comp. Neurol.*, 1938, **68**, 411.

from area 6 or even from the rostral part of area 4. According to them, 80% of the fibers come from area 4 and the remainder from areas 3, 1, 2, and 5 in the parietal lobe.

Lassek and Rasmussen[5] and Lassek[6] who first conclusively showed that the Betz cell is an unreliable criterion of the origin of the pyramidal tract. Lassek's subsequent work has confirmed and extended his original findings and it is now generally admitted that only a small part of the corticospinal system can be traced to such cells. Do the numerous non-Betz origin fibers arise from smaller cells in the region where the large cells are found? Do they come from other cortical regions (Peele[7] was of the opinion that they arose even from the back of the parietal lobe)? Is it true, as Marie and Guillain[8] suggested, that fibers, which they called parapyramidal, arising in the brain stem, enter the pyramids?

The answer which one gives to these questions will depend, to some extent, upon the techniques employed to study them and we may divide our observations upon such a basis.

Cell-degeneration Technic. If one cuts the corticospinal system and searches for chromatolytic or, if the degeneration time is longer, grossly abnormal cells in the cortex, it is very difficult to draw conclusions as to whether any particular cell is abnormal or to find such cells if they are dispersedly arranged. Moreover, doubt must always be entertained as to whether the obviously abnormal cells are specifically related with damage of the corticospinal system and nothing else. The present writer has not been able to draw any specific conclusions upon the origin of the non-Betz pyramidal fibers, from such material.

Marchi Method. If area 4 is removed and any of the Marchi methods are employed the amount of degeneration in the pyramids is so extensive that scarcely any normal fibers can be found. Obviously one cannot conclude from such evidence that some pyramidal fibers do not arise outside area 4.

If areas other than 4 are removed, few if any degenerated fibers are found in the pyramids of Marchi-stained material. There are exceptions to this rule. If a ledge of bone is allowed to press on area 4, if burrs are used in its vicinity, or if it is closely approached or the dura incorrectly sutured, true degeneration may result from that region even though not directly operated upon. Moreover, if the Swank-Davenport technic is employed, and occasionally with any Marchi method, a certain amount of degeneration may be found in apparently normal material. This may represent casual, inevitable pathology or be clearly artifactual in nature. Negative evidence, under such circumstances, means more than positive and, in the present writer's experience, no conclusive data of this type has been encountered to indicate that true degeneration of the pyramids ever results from lesions which are far from area 4.

Sheath Stains. If myelin stains of the classic type are used, no intact fibers can be seen in the pyramids if area 4 is removed and 6 months or so are allowed to elapse. If, however, one employs stains such as the Weil or Mahon, falsely positive results are obtained within such a degeneration period. These range all the way from the staining of a few, scattered fibers of variable caliber to a condition in which the entire pyramid is well though differently stained from the normal. Here again, then, negative are preferable to positive findings and the results agree with what one obtains with the Marchi technic.

Silver Stains. Most of the above difficulties are obviated by the use of any reliable silver technic. When such procedures are employed, and sufficient time for degeneration is allowed to elapse, markedly different results are seen after ablations of different cortical areas. Thus, if only the caudal parts of area 4 and the adjacent portion of area 3 are ablated (case J 141, the physiologic features of which have been separately reported[9]), practically all the large fibers in the pyramids disappear but the smaller, thinly-myelinated and non-myelinated fibers remain. If one examines silver sections taken from congenitally abnormal brains, in which the telencephalon is largely absent but the infratentorial regions normal, no trace of a pyramidal system can be detected.[10] It is obvious, therefore, that Marie and Guillain's conjecture that fibers from the brain stem itself contribute to the pyramidal system must be incorrect, at least with regard to infratentorial levels.

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[5] Lassek, A. M., and Rasmussen, G. L., *Arch. Neurol. and Psychiat.*, 1939, **42**, 872.

[6] Lassek, A. M., *Arch. Neurol. and Psychiat.*, 1940, **44**, 718.

[7] Peele, T. L., *Anat. Rec.*, 1942, **82**, 44.

[8] Marie, P., and Guillain, G., *Rev. Neur.*, 1904, **12**, 697.

[9] Mettler, Fred. A., *J. Comp. Neurol.*, 1944, **81**, in press.

We may now ask whether the diencephalon contributes any fibers to the system. If all the cortex and corpus striatum are removed from an animal (case J 31, the physiologic, cytologic, and myelinated fiber features of which have been previously reported[11]) silver stains again reveal no fibers in the pyramids. The same is true of silver examinations made upon a monkey lacking only cortex and putamen (case J 33, other studies of which have been published[12]). We can, in fact, state that the small pyramidal fibers are definitely of cortical origin for silver studies of the pyramids of our case 36, from which only cortex had been removed,[13] have also failed to disclose any sound pyramidal fibers in silver section.

Conclusion. Practically all the heavily myelinated fibers in the pyramids of the monkey's brain arise from the region of the cerebral cortex in which the gigantopyramidal cells of Betz are found. Since we know that all of these fibers cannot be accounted for as arising from such cells they must also come from smaller elements. The fact that Weigert material fails to show any fibers in the pyramids after ablation of the precentral gyri, does not prove that the pyramids are devoid of fibers for smaller elements can plainly be seen with silver stain. Since no fibers remain after removal of all of the cortex none of these smaller fibers can be attributed to an infrapallial origin.

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[10] Marburg, O., and Mettler, Fred. A., *J. Neuropath. and Exp. Neur.*, 1943, **2**, 54.

[11] Mettler, Fred. A., *J. Comp. Neurol.*, 1943, **79**, 209.

[12] Mettler, Fred. A., *J. Comp. Neurol.*, 1943, **79**, 204.

[13] Mettler, Fred. A., *J. Comp. Neurol.*, 1943, **79**, 195.

14718 P

Effect of Pyridoxine on Activity of Quinine and Atabrine Against *Plasmodium lophuræ* Infections.

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From the Merck Institute for Therapeutic Research, Rahway, N.J.

In an investigation of the effect of large amounts of vitamins on the activity of quinine and atabrine in avian malaria it was found that massive doses of pyridoxine markedly inhibited the activity of these drugs against *Plasmodium lophuræ* infections.

Pekin ducklings weighing about 50 g each were inoculated intravenously with 10,000,000 erythrocytes parasited by *P. lophuræ*. The ducks were allowed to feed at will on a commercial starting mash. In all experiments quinine sulfate and atabrine dihydrochloride were administered as aqueous solutions into the crops by tube once daily for 6 days beginning on the day of inoculation. Pyridoxine hydrochloride was given in the same manner in most of the experiments but as is indicated on the accompanying tables it was in certain cases administered subcutaneously. Since both quinine and pyridoxine are excreted quite rapidly they, although given individually, were always administered within an interval of about 30 minutes. Blood smears were made on the seventh day after inoculation and the number of parasitized erythrocytes per 10,000 erythrocytes was determined.

Pyridoxine when given in a large daily dose produced almost complete inhibition of the

TABLE I.
Effect of Pyridoxine on Activity of Quinine Against the Schizonts of *P. lophuræ.*
5 ducks in each experiment.

Exp. No.	Drug	Dose, mg/kg	Route	% of erythrocytes parasitized on 7th day
1	Quinine	15	p.o.	1.5
	Quinine +	15	p.o.	33.2
	Pyridoxine	500		
	Pyridoxine	500	p.o.	46.5
	Controls	—	—	51.1
2	Quinine	20	p.o.	1.5
	Quinine +	20	p.o.	33.8
	Pyridoxine	500		
	Pyridoxine	500	p.o.	32.7
	Controls	—	—	40.8
3	Quinine	10	p.o.	0.3
	”	15	p.o.	0.02
	Quinine +	10	p.o.	39.1
	Pyridoxine	200	s.c.	
	Quinine +	15	p.o.	5.9
	Pyridoxine	200	s.c.	
	Pyridoxine	200	s.c.	74.2
	Controls	—	—	66.5

TABLE II.
Effect of Pyridoxine on Activity of Atabrine Against the Schizonts of *P. lophuræ.*
5 ducks in each series.

Exp. No.	Drug	Dose, mg/kg	Route	% of erythrocytes parasitized on 7th day
1	Atabrine	5	p.o.	2.68
	Atabrine +	5	p.o.	48.7
	Pyridoxine	500		
	Pyridoxine	500	p.o.	46.5
	Controls	—	—	51.1

activity of quinine and atabrine at the dose levels used (Table I and II). The doses of quinine and atabrine used in these experiments were selected because experience had shown them to be approximately the minimal amounts necessary for suppression of the disease. The dose of pyridoxine was chosen arbitrarily and although the exact requirement is not known it probably represented several thousand times the amount of vitamin needed by the duckling for optimal growth.

When pyridoxine was administered subcutaneously at 200 mg per kg it markedly inhibited the activity of a 10 mg per kg dose of quinine but was only moderately effective against a 15 mg per kg dose of quinine (Table I). This experiment showing that the inhibiting power of a unit dose of pyridoxine varies with the amount of quinine involved suggests that there may be a quantitative relationship but the exact nature of this relation remains for further study.

In the experiments in which both the antimalarials and pyridoxine were given by mouth within a short interval it was conceivable that the inhibition might have been due to a direct interaction in the intestinal tract before complete absorption had occurred. This explanation seems unlikely in view of the experiment in which the pyridoxine was shown to have inhibiting power even when it was administered subcutaneously and the drug given by mouth.

The fact that pyridoxine inhibits the action of quinine and atabrine on *P. lophuræ in vivo* suggests the possibility that pyridoxine or a compound of similar structure is essential for the parasite and that quinine and atabrine act by competing with a pyridoxine-like compound for a position in some enzyme system of the parasite. Interestingly, Silverman and Evans[1] have reported that while certain polyamines will antagonize the growth inhibitory action of atabrine on *Escherichia coli in vitro* pyridoxine under their experimental conditions failed to effect the action of atabrine.

Summary. Pyridoxine when administered in doses several thousand times greater than required for the nutrition of the host will inhibit the activity of minimal effective doses of quinine and of atabrine against *P. lophuræ* infections in Pekin ducklings.

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[1] Silverman, Milton, and Evans, E. A., Jr., *J. Biol. Chem.*, 1943, **150**, 265.

14719

Elevated Plasma Fibrinogen Levels and Liver Necrosis in Rats Receiving Atabrine.*

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Wright and Lillie[1] and Siegel and Mushett[2] have reported the appearance of necrotic areas in the livers of rats receiving atabrine. Scudi and Hamlin[3] studied the effect of atabrine on dogs and rats fed purified diets varying in protein content and suggested that an increase in plasma fibrinogen in their animals was a result of liver damage. This study has been extended to establish the correlation between the increase in plasma fibrinogen and the presence and extent of liver damage in individual rats dosed with atabrine.

Experimental and Results. Ninety-two rats, weighing 120 to 250 g each, were given 150 mg of atabrine per kg by stomach tube three times weekly. At intervals of 1 to 4 weeks, rats were sacrificed and examined for liver necrosis. Plasma fibrinogen levels of all animals, including 75 untreated controls, were determined by the method of Cullen and Van Slyke.[4]

The plasma fibrinogen levels of these rats were as follows: 75 untreated controls averaged 212.5 ± 4.2[†] mg % (range 135-300); 50 atabrine dosed rats showing no evidence of gross liver necrosis, 232 ± 4.8 mg % (range, 160-320); and 42 atabrine-dosed rats which had gross liver necrosis, 384.4 ± 12.3 mg % (range 240-570). Thus, in the treated group which did not show grossly necrotic livers there was a slight but significant increase in plasma fibrinogen over the level found in the untreated controls. A much more striking elevation in plasma fibrinogen accompanied the development of gross liver necrosis.

The body weight of the rats appeared to be a predisposing factor in the development of liver necrosis since the damage occurred earlier in large rats, as shown in Table I.

In the group of rats with an average initial weight of 120 g (Table I), dosed 3 times weekly, liver necrosis began to develop during the 6th week. In a second group of rats of the same initial weight but receiving only 2 doses per week, liver necrosis became apparent during the 6th week even though these animals received only two-thirds the total number of doses administered to the first group. This may be explained by the fact that the blood levels of both groups averaged about 5 γ per cc. Therefore, in these rats, the occurrence of liver necrosis was more a function of

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[*] The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Merck Institute for Therapeutic Research.

[1] Wright, C. I., and Lillie, R. D., *Public Health Rep.*, 1943, **58**, 1242.

[2] Siegel, H., and Mushett, C. W., in press.

[3] Scudi, J. V., and Hamlin, M., *J. Pharm. and Exp. Therap.*, 1944, **80**, 150.

[4] Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, **41**, 587.

[†] Standard error.

TABLE I.
Liver Necrosis and Fibrinogen Levels in Rats of Different Weights Dosed with 150 mg of Atabrine per kg 3 Times per Week.

Initial wt. in g	No. of rats	No. of doses	Incidence of liver necrosis, %	Avg level of fibrinogen, mg %
120	15	10	0	220
155	19	9	5	260
180	5	8	60	325
210	7	8	100	440
245	7	8	100	550

time than total dose of atabrine.

With prolonged dosing, the liver necrosis became more extensive and the plasma fibrinogen levels higher. Thus, in the above group which was dosed twice weekly, those rats showing necrotic livers after 5 weeks had an average fibrinogen level of 300 mg %; after 9 weeks, 365 mg %; and after 10-13 weeks, 460 mg %.

Discussion. Foster and Whipple[5] have shown that, although certain types of liver damage, such as that induced by chloroform or phosphorus, depress plasma fibrinogen levels, the level is elevated in a variety of other pathological conditions. They state that " . . . tissue injury unaccompanied by the usual inflammatory reaction is sufficient to give this specific physiological reaction—an overproduction of fibrin in the body." However, in their study of plasma fibrinogen levels in animals with damaged livers, elevated fibrinogen levels were observed only after very limited exposure to chloroform or phosphorus. Prolonged treatment invariably resulted in depressed fibrinogen levels. In contrast to this condition, the atabrine-dosed rats described in this paper showed no decrease in fibrinogen levels. A slight increase was found prior to the development of gross areas of necrosis in the liver. The histopathological picture at this stage, namely, early degenerative changes preceding gross liver necrosis, could easily be compared with that found in the early stages of liver damage caused by chloroform or phosphorus.[2] With prolonged dosing this necrotic condition became more extensive and was accompanied by correspondingly increased plasma fibrinogen levels, with maximal levels obtained when as much as two-thirds of the liver were involved, although in advanced stages of liver poisoning by other agents the plasma fibrinogen levels are low.

The earlier appearance of necrotic areas in the livers of large rats raised the question of the validity of toxicity comparisons in rats of different weights, dosed on a body weight basis. Since the liver is the principal organ damaged by the administration of atabrine, it seemed possible that a change in the ratio of liver weight to body weight might account for this difference in toxicity. The livers of rats weighing 100 to 107 g constituted approximately 4.3% of their body weight; those of 180 to 225 g rats, 3.2%; and of 250 to 465 g rats, 2.5%. Consequently, when doses of 150 mg per kg were administered, a 100 g rat actually received 3.5 mg per g liver whereas a 300 g rat received 6 mg per g liver. It is not surprising that under these conditions the livers of heavy rats developed necrosis first.

Summary. The increase in plasma fibrinogen levels in rats receiving atabrine has been correlated with the presence and degree of necrosis in the liver.

The maintenance of a blood level of about 5 γ per cc in 120 g rats was sufficient to induce gross liver damage after 5 weeks, irrespective of the number of doses given.

When dosed on a mg per kg basis, older rats appeared to have a decreased tolerance to atabrine poisoning. Evidence has been presented to show that the decrease in the ratio of liver weight to body weight with age may account for the earlier onset of liver damage in older rats.

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[5] Foster, D. P., and Whipple, G. H., *Am. J. Physiol.*, 1922, **58**, 407.

14720

Antipressor Effect of 1,4-Cyclohexandione on Blood Pressure of Hypertensive Dogs.*

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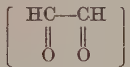
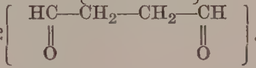
In a previous communication it was shown that some of the quinones are effective in lowering the blood pressure of hypertensive rats.[1] In the course of extending these observations to hypertensive dogs it was noted that, although some antipressor activity is possessed by the quinones, these chemicals were extremely toxic and their use sometimes resulted in the death of the treated dog.[2] It has recently been demonstrated that one of these quinones (xyloquinone) is effective in lowering the blood pressure of the dogs with "neurogenic" hypertension, but in larger doses produced hemolysis, tubular casts, uremia, and death.[3] Other work as well as our own has indicated an antipressor effect on hypertensive rats of the well-known quinone vitamin K (menadione).[4]

It was believed by us, however, that a further search should be made among compounds related to the quinones for antipressor agents with less toxicity. The quinones may, for our purpose, be regarded as highly reactive forms of unsaturated cyclic diketones. Accordingly, a search was begun among the carbonyl compounds (both aldehydes and ketones) for further antipressor agents.[2] It was quickly noticed that *mono*carbonyl compounds (aldehydes, ketones, and cyclic ketones) were inactive. Among the *di*carbonyl compounds (dialdehydes and diketones) certain of the shorter, straight-chain dialdehydes were able to reduce the pressure of both hypertensive rats and hypertensive dogs, but were extremely toxic to both animals. In this group belong glyoxal  $\left[\underset{\mathrm{O}}{\underset{\|}{\mathrm{HC}}}{-}\underset{\mathrm{O}}{\underset{\|}{\mathrm{CH}}}\right]$ and succinic dialdehyde  $\left[\underset{\mathrm{O}}{\underset{\|}{\mathrm{HC}}}{-}\mathrm{CH_2}{-}\mathrm{CH_2}{-}\underset{\mathrm{O}}{\underset{\|}{\mathrm{CH}}}\right]$. The shorter chain compound was more active and more toxic than the longer chain dialdehyde. The 6-carbon analogue (adipic dialdehyde) was quite inactive. The straight-chain diketones were without exception inactive.

Inasmuch as the dialdehydes were active and the diketones were not, it seemed reasonable to suppose that the presence of two carbonyl groups was essential for activity; but that the effect depended upon the fact that the carbonyl groups in the quinones and in the aldehydes are more active than those present in the diketones. Inasmuch as cyclic diketones are somewhat more closely related to quinones than are open chain diketones and dialdehydes, it was considered desirable to investigate the activity of the cyclic diketones 1,2- and 1,4-cyclodexandione. Since 1,2-cyclohexandione is inactive in hypertensive rats as reported by us in a previous paper,[2] this compound was not investigated for its effect in dogs.

The *methods* previously described[1] for producing hypertension in the dog were again employed. In most experiments the hypertension was produced by the use of the Gold-

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[1] Friedman, Ben, Soloway, Saul, Marrus, Joseph, and Oppenheimer, B. S., Proc. Soc. Exp. Biol. and Med., 1942, **51**, 195.

[2] Oppenheimer, B. S., Soloway, Saul, and Lowenstein, B. E., *J. Mount Sinai Hosp.*, 1944, **11**, 23.

[3] Schafer, Paul W., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 274.

[4] Schwarz, Harry, and Ziegler, William M., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 160.

TABLE I.
Responses of Hypertensive Dogs to Intramuscular Injections of 1,4-Cyclohexandione.

Dog No.	Duration of hypertension before treatment, months	Blood pressure before treatment, mm Hg	Daily dose of 1,4-cyclohexandione, mg/kg/day	Period treated, days	Blood pressure after treatment, mm Hg	Fall in blood pressure, mm Hg	Latency of response, days	Interval before return of blood pressure to hypertensive level, days
177	24	180/115	10	7	135/90	45/25	2	15
204	15	180/120	4-22	12	175/120	5/0	—	—
216	14	190/120	7	9	147/70	43/50	3	6
218	14	190/127	7	9	153/100	37/27	?	3
219	14	185/120	6-24	12	163/110	22/10	5	10
177	27	178/110	10	7	140/74	38/36	3	12
204	18	190/110	”	”	157/75	33/35	3	5
216	17	181/107	”	”	148/77	33/30	3	8
218	17	182/110	”	”	162/80	20/30	3	11
219	17	188/106	”	”	160/82	28/24	6	11
239*	1	170/102	”	”	155/80	15/22	2	3
240†	2	180/108	”	”	144/74	36/34	3	—
Avg		183/113			153/86	30/27	3.6	7.4

* Dog 239 was made hypertensive by a Goldblatt clamp on left renal artery and cellophane wrapping of the right kidney.

† Dog 240 was made hypertensive by the injection of croton oil into left renal artery and subsequent application of a Goldblatt clamp to the right renal artery.

blatt clamp, but in one dog an emulsion of croton oil was twice injected into the left renal artery and subsequently the right renal artery was clamped, and in still another dog the right kidney was wrapped in cellophane according to the method of Page and the left kidney was clamped. The blood pressure was measured by the indirect auscultatory method using a sphygmomanometer cuff and mercury manometer. The dog was considered hypertensive when the high blood pressure was maintained for one month or more. The drug, which is quite soluble, was dissolved in distilled water and injected intramuscularly into the lumbar muscles. Blood pressures were taken daily, but were omitted on some holidays. The dose of 1,4-cyclohexandione varied from 4-24 mg per kg per day. The blood pressures recorded in Table I are the separate medians of the systolic and the diastolic pressure for the 5 days preceding the treatment, and for the period during which the antipressor response was maintained.

Results. The results are shown in Table I. The experiments have been divided into 2 series. In the first series 5 hypertensive dogs were injected with varying doses of 1,4-cyclohexandione, and in the second the same 5 dogs with the addition of 2 other hypertensive dogs were each given 10 mg per kg of body weight daily for 7 days. Although one of the five dogs failed to respond and another gave only a moderate antipressor response in the first series, all the dogs including these 2 responded well in the second series in which the optimal dose was used.

Evidently the compound, 1,4-cyclohexandione, had an antipressor effect in these hypertensive dogs. It should be noted that the blood pressure did not fall immediately after the administration of the compound, but that a latent period of 3 to 7 days intervened in all cases. Moreover, after injections were stopped, a similar but somewhat longer interval elapsed before the blood pressure returned to the pre-treatment level.

The intramuscular injections in hypertensive dogs were apparently painless and produced no gross evidence of local or general toxic reactions. Large doses, many times the optimal therapeutic dose, injected into normal rats and normal dogs had no effect on the blood pressure, and gave no evidence of toxicity in either species.

The administration of 10 mg per kilo of the same compound to 2 normal dogs for 6 days did not affect their blood pressure.

Discussion of Results. Our previous results on hypertensive rats have shown that 1,2-cyclohexandione is inactive, but that 1,4-cyclohexandione possesses moderate antipressor activity. Although 1,4-cyclohexandione is much less active than some of the quinones previously investigated, it is so much less toxic to rats and dogs that it offers greater promise as an antipressor agent than any of the other 34 compounds we have studied thus far.

The mode of action of all these antipressor agents is still obscure. The antipressor activity of the dicarbonyl compounds declined in the order, *p*-quinones, *o*-quinones, dialdehydes, cyclic diketones, straight-chain diketones, and their toxicity decreased in the same order. The cyclic diketones are the first group of compounds in this series which are not too toxic for use.

Summary. Daily intramuscular injections for one week of the diketone, 1,4-cyclohexandione, into hypertensive dogs resulted in a moderate reduction of blood pressure. In the dosage thus far employed there has been no gross evidence of local or general toxic effect.

14721

An Improved Method for Testing of Bacteriostatic Agents Using Virulent Human Type Tubercle Bacilli.*

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The demonstration by Feldman and his co-workers[1-3] and others[4,5] of the therapeutic effect of various compounds on experimental tuberculosis of guinea pigs increases the necessity for a rapid and accurate *in vitro* method for testing the relative growth inhibitory powers of compounds on *M. tuberculosis var. hominis*.

Several investigators[5-15] have used virulent human tubercle bacilli for this purpose, employing a variety of media both liquid and solid. The use of complex solid or liquid media is unsatisfactory because of the possible presence of antagonists; agar when added to a synthetic medium is in itself slightly inhibitory.[16] The use of liquid synthetic media has previously required relatively large volumes of medium in flasks, to provide sufficient surface growth, inoculated on the surface with flakes of pellicle growth. The results are usu-

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* This work was aided by a grant from Parke Davis and Co., Detroit, Mich.

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[2] Feldman, William H., and Hinshaw, H. Corwin, *Am. J. Clin. Path.*, 1943, **13**, 144.

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[12] Rist, Noel, *C. R. Soc. de biol.*, 1939, **130**, 972.

[13] Middlebrook, Gardner, and Lloyd, John B., *Am. Rev. Tuberc.*, 1944, **49**, 535.

[14] Middlebrook, Gardner, and Lloyd, John B., *Ibid.*, 1944, **49**, 539.

[15] Saz, Arthur K., and Bernheim, Frederick, *J. Pharm. and Exp. Therap.*, 1941, **73**, 78.

[16] Drea, W. F., *J. Bact.*, 1940, **39**, 197.

ally read over a period of 4-8 weeks in terms of inhibition of pellicle growth. The above methods are cumbersome and time consuming, do not permit the use of a uniform inoculum and the results are difficult to read.

These technical difficulties have led several investigators[17-19] to substitute rapidly growing avirulent acid-fast bacilli as test organisms. The validity of the use of such organisms for this purpose would depend upon there being a correlation between the inhibitory action of chemotherapeutic compounds upon the virulent and avirulent strains. So far this correlation has been assumed without being demonstrated.

The demonstration by Drea[16,20] and by Youmans[21] that suspensions of virulent human tubercle bacilli will grow rapidly beneath the surface and eventually form pellicle growth when inoculated into a synthetic medium permits the utilization of these virulent bacilli as test organisms in the same manner that avirulent rapidly growing acid-fast organisms have hitherto been employed.

Methods. In view of the fact that the H37Rv strain has been extensively utilized as the test organism for *in vivo* work[1-3] it was chosen as the *in vitro* virulent test organism. For comparison we chose National Type Culture Collection organism 607, an avirulent rapidly growing acid-fast bacillus, employed by both Fitzgerald and Feinstone[19] and Freedlander[17,18] as an *in vitro* test organism. Suspensions were made as previously described[21] from 21-day-old surface growths except that in addition all cultures were washed 3 times with double distilled water before suspensions were made. The medium employed was the synthetic one previously described.[21†] Suspensions were standardized by centrifugation in Hopkins vaccine tubes.

The compounds used were 4,4′diaminodiphenyl sulfone, sulfanilamide, sulfathiazole, and sulfadiazine. These were diluted serially, starting with concentrations of 10 mg %, each dilution being half the preceding one. Separate pipettes were used for each dilution. The final volume of each concentration was 10.0 cc and sterilization was accomplished by autoclaving at 10 lb for 20 minutes. Each tube was inoculated, including suitable controls, with 0.1 cc of suspension containing the desired amount of tubercle bacilli. Readings of growth inhibition of the rapidly growing avirulent 607 strain were made at 7 days, and of the slower growing virulent H37Rv culture at 21 days. The latter cultures were shaken at 7 and 14 days since more regular development of surface growth occurred in the controls following this treatment. Readings were made by comparing the growth in the tubes containing the drug with the growth present in medium containing no drug. All tests were run in duplicate and were usually found to check within limits (plus or minus) of one dilution when using the avirulent 607 strain. With the H37Rv culture greater variations were sometimes encountered. Since it was observed that the growth-inhibiting concentrations were not identical for surface and subsurface growth, separate recordings were made for each.

Results. The lowest concentrations necessary to completely inhibit subsurface growth were usually higher than those necessary to completely inhibit surface growth. (Table I.)

The amount of inoculum used was important. With inocula of 1.0 mg H37Rv and 0.1 mg 607 there was subsurface growth in all of the concentrations of all 4 compounds, whereas surface growth was completely inhibited at some point in the series. One-tenth mg H37Rv and 0.01 mg 607 usually gave endpoints of complete growth inhibition for both surface and subsurface growth. Smaller inocula of H37Rv were unsatisfactory because of the prolonged growth period and irregular

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[17] Freedlander, B. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 153.

[18] Freedlander, B. L., *Am. Rev. Tuberc.*, 1944, **49**, 543.

[19] Fitzgerald, Robert S., and Feinstone, W. Harry, PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 27.

[20] Drea, W. F., *J. Bact.*, 1943, **44**, 149.

[21] Youmans, Guy P.

[†] The troublesome precipitates sometimes encountered on autoclaving this medium can be prevented by substituting K_2SO_4 for the $MgSO_4$ in the formula. Growth of the organisms is not affected by this substitution.

results, whereas smaller inocula of 607 behaved in much the same manner as the 0.01 mg inoculum.

TABLE I.
Lowest Molar Concentrations of Compounds Completely Inhibiting Surface and Subsurface Growth of H37Rv and 607 and the 4,4′diamino diphenyl sulfone Coefficients.

	607		H37Rv	
	Molar concentration	Coefficient	Molar concentration	Coefficient
	Subsurface Growth			
4,4′diamino diphenyl sulfone	2.20×10^{-4}	1.0	2.82×10^{-5}	1.0
Sulfanilamide	$>2.18 \times 10^{-4}$	< 1.02	$>1.16 \times 10^{-3}$	<0.0244
Sulfathiazole	5.32×10^{-6}	41.5	3.06×10^{-5}	0.927
Sulfadiazine	1.34×10^{-5}	17.7	1.04×10^{-4}	0.272
	Surface Growth			
4,4′diamino diphenyl sulfone	2.34×10^{-5}	1.0	6.28×10^{-6}	1.0
Sulfanilamide	1.45×10^{-4}	0.161	5.36×10^{-4}	0.0144
Sulfathiazole	3.06×10^{-6}	7.47	7.64×10^{-6}	0.824
Sulfadiazine	1.24×10^{-5}	1.87	3.74×10^{-5}	0.168

Table I shows the molar concentrations of the 4 compounds which completely inhibited surface and subsurface growth of both H37Rv and 607 using 0.1 and 0.01 mg inocula respectively. These figures are averages of 6 separate duplicate tests run at different times. In addition, the table gives the "4,4′diaminodiphenyl sulfone coefficient," calculated by dividing the inhibiting molar concentration of this drug by the inhibiting molar concentrations of the other compounds. This gives the relative effectiveness of these compounds in inhibiting growth as compared with the effectiveness of diaminodiphenylsulfone.

None of the compounds inhibited equally the growth of the two organisms. Sulfathiazole and sulfadiazine were far more effective in inhibiting the growth of M. 607 than was 4,4′diaminodiphenylsulfone, although they were somewhat less effective, relatively, in inhibiting surface growth of this organism, than subsurface growth. On the other hand both surface and subsurface growth of H37Rv was more effectively inhibited by 4-4′diaminodiphenylsulfone than by any of the others although sulfathiazole was almost equal to it in effectiveness.

Fitzgerald and Feinstone[19] also found that sulfathiazole and sulfadiazine were more effective in inhibiting growth of 607 than 4,4′diaminodiphenylsulfone. However, they found sulfadiazine and sulfathiazole equally effective in inhibiting growth of this organism whereas we have consistently found sulfathiazole to be more effective than sulfadiazine. Smith and Emmart[5] using the moderately virulent H37 strain also reported that sulfathiazole and sulfadiazine were less effective than 4,4′diaminodiphenylsulfone in preventing surface growth.

The significance of the results reported in this paper is more striking when it is considered that of the 4 compounds used 4,4′diaminodiphenylsulfone is the only one which shows marked chemotherapeutic action on experimental tuberculosis of guinea pigs produced by H37Rv.[22] This clearly illustrates the possible fallacy involved in using an avirulent rapidly growing acid-fast organism for *in vitro* testing of potential chemotherapeutic agents for tubercle bacilli of human type.

The technic described has the following advantages over those previously described: (1) it is less cumbersome; (2) permits the use of virulent human type of tubercle bacilli; (3) it results in a more homogeneous inoculum; and (4) permits the use of a uniform inoculum for all tubes; (5) makes results easier to read; (6) reduces the time necessary for completion of the tests.

Results obtained with any one compound not infrequently differ from time to time, probably due in the main to two factors: (1) difficulty in preparing suspensions of the same homogeneity or degree of dispersion, and (2) biological variation in cultures used for inoculation even though cultures of the same age are employed. These factors can be controlled by including in the test, as a control, a standard compound such as 4,4′diaminodiphenylsulfone each time unknown compounds are tested and evaluating the results in terms of this as a standard. Improvements in the methods of preparing suspensions and in determining the amount of inoculum would also be helpful. For example, in a limited number of tests, micro-Kjeldahl nitrogen determinations have proved to be far more accurate for the latter purpose than Hopkins vaccine tubes.

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[22] Feldman, W. C., Hinshaw, H., and Moses, H. E., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 60.

The time of the completion of the tests can be reduced to 7-14 days providing only the readings of subsurface growth are recorded and a slightly larger inoculum employed.

Summary. A new technic for rapidly and efficiently testing the growth-inhibiting action of substances on virulent human type tubercle bacilli is described. The order of effectiveness of four compounds in inhibiting the growth of the avirulent acid-fast rapidly growing organism 607 was found to be: Sulfathiazole, sulfadiazine, 4,4′diaminodiphenylsulfone, and sulfanilamide, while for the virulent human strain of tubercle bacillus H37Rv the order was found to be: 4,4′diaminodiphenylsulfone, sulfathiazole, sulfadiazine, and sulfanilamide.

14722 P

Subsurface Growth of Virulent Human Tubercle Bacilli in a Synthetic Medium.*

GUY P. YOUMANS. (Introduced by Alex. A. Day.)

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While there are scattered publications reporting growth of virulent human tubercle bacilli beneath the surface of liquid media, it is generally believed that these organisms will grow only on the surface, and precautions are always taken to float the organisms when subculturing. Drea[1,2] reported that one strain (H37) would grow rapidly in the depths of Long's synthetic medium when suspensions containing as little as 10^{-6} to 10^{-8} mg were inoculated. These findings with H37 have recently been confirmed by Cohn.[3] The present paper reports subsurface growth in a synthetic medium of 5 other virulent human strains of tubercle bacilli and one avirulent rapidly growing acid-fast organism.

Methods. Five virulent human strains were employed: H37Rv, 3637,[†] 3632,[†] G-2,[‡] and G-5.[‡] All 5 strains were pathogenic for guinea pigs. H37Rv, G-2, and G-5 were also pathogenic for mice on intravenous inoculation, and G-2 has been used to produce pulmonary tuberculosis in dogs.[4] No. 607, an avirulent rapidly growing acid-fast organism, was obtained from the National Type Culture Collection.

A synthetic medium was employed containing: Asparagin, 0.5%; monopotassium phosphate, 0.5%; magnesium citrate, 0.25%; magnesium sulfate, 0.05%; glycerol, 2.0%, made up with water redistilled from glass. The pH was adjusted to 7.0 with 40% sodium hydroxide. This medium was tubed in the desired amount, usually 10.0 cc, in 200 × 25 mm new soft glass or pyrex test tubes and sterilized by autoclaving at 10 lb for 20 minutes.

Suspensions of tubercle bacilli were pre-

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[*] This work was aided by a grant from Parke Davis and Co., Detroit, Mich.

[1] Drea, W. F., *J. Bact.*, 1940, **39**, 197.

[2] Drea, W. F., *J. Bact.*, 1942, **44**, 149.

[3] Cohn, Maurice L., *Am. Rev. Tuberc.*, 1944, **49**, 463.

[†] Obtained from Dr. William H. Feldman, Mayo Clinic, Rochester, Minn.

[‡] Isolated from patients with active pulmonary tuberculosis by Dr. Francis D. Gunn, Department of Pathology, Northwestern University Medical School.

[4] Mills, Moore A., Barth, E. E., and Gunn, F. D., *Am. Rev. Tuberc.*, 1940, **48**, 28.

pared by grinding aseptically 21-day surface growths from synthetic medium in an agate mortar and suspending them in either sterile double distilled water or in the synthetic medium. No dispersing agent was employed. These suspensions were then standardized by centrifugation in Hopkins vaccine tubes. For inoculation 0.1 cc volumes, containing the desired amount of tubercle bacilli, were used and incubation was at 37°C.

Results. With inocula of 1.0 mg in 10.0 cc of medium, growth was always evident by increased turbidity within 2 days, this rapidly increased and consisted of growth of tubercle bacilli occurring between the medium and the side of the tube. When the tubes were shaken flaky masses of tubercle bacilli could be seen which settled to the bottom where they continued to grow. Six to 10 days following the inoculation the familiar pellicle development begins to take place. With smaller inocula (0.1 mg) subsurface growth may not be evident for from 6 to 10 days and surface pellicle development may not appear until 10 to 14 days following inoculation. With inocula of 0.01 and 0.001 mg subsurface growth usually was not evident until from 2 to 4 weeks and in some tubes did not appear at all within this time.

The degree of dispersion of the organisms in the suspensions employed for inoculation was important in influencing the rapidity with which subsurface growth appeared. The more finely divided suspensions seemed to grow more rapidly than those consisting of larger clumps of bacilli. Whether the actual amount of growth would be different in the two cases, however, has yet to be determined.

That the growth evident in the cultures actually consisted of tubercle bacilli is indicated by the fact that stains made from them consistently showed only masses of acid-fast bacilli, and subcultures made on a variety of media incubated both aerobically and anaerobically were consistently negative for any organisms other than tubercle bacilli. Contamination, which occasionally occurred, was always grossly evident, and on stain and subculture revealed the usual laboratory contaminants. That the organisms after growing as indicated above are still virulent was demonstrated by the fact that a 3-week-old subsurface growth of H37Rv produced progressive tuberculosis when inoculated into guinea pigs.

The same phenomena of subsurface growth can be observed in 250 cc flasks containing 100 cc of medium; in fact, if the inoculum which is usually floated so carefully on the surface of the medium is allowed to sink and the flask than shaken vigorously to disperse the organisms, subsurface growth is usually evident within a few days. If the flask is not shaken further the usual surface pellicle growth will eventually appear. However, if the flask is shaken daily pellicle development will be prevented, and only subsurface growth will occur.

All 5 virulent strains of human type tubercle bacilli grew as described above and while differences have been noted between strains in the rate of subsurface growth the significance of this is not known since individual strains varied in their rate of growth at different times.

When 0.1 mg of a suspension of the avirulent strain No. 607 was inoculated into 10.0 cc of synthetic medium, surface pellicle growth usually appeared within 2 to 3 days. However, if prior to this time the tubes were shaken subsurface growth could be detected. In our experience with this strain subsurface growth has always appeared before surface growth.

The above results show that many, if not all, human type tubercle bacilli are capable of rapidly developing beneath the surface of liquid synthetic medium provided a sufficiently large inoculum is used. In fact, subsurface growth may be the more normal type of growth since, as pointed out by Drea,[1,2] it is more analogous to the type of growth which takes place *in vivo* than is pellicle development.

The ease and rapidity with which tubercle bacilli will grow in this manner in a synthetic medium should remove many of the technical difficulties which have confronted investigators working with these organisms. It should provide a far more rapid and accurate, and a far less cumbersome, method for studying the metabolism of human type tubercle bacilli, and for the *in vitro* testing of growth promoting or growth inhibiting substances.

Summary. Suspensions of five virulent strains of *My. tuberculosis var. hominis* were found to rapidly produce subsurface growth when inoculated into synthetic medium. One avirulent rapidly growing strain grew in a similar manner.

14723 P

Response of Rats to Diets Containing Propylene Glycol and Glycerol.

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The present paper is a brief preliminary report regarding some of the principal observations in connection with experiments which were started in 1941 in an attempt to compare the physiological effects of propylene glycol and glycerol when fed to rats at different levels of intake.

The first series of growth experiments covered a period of 20 weeks. The basal ration consisted, in parts per 100, of casein 20, salt mixture 4, Cell-U-Flour 2, butter fat 6, cod liver oil 2, brewer's yeast 6, and corn starch 60. Each experimental group consisted of 6 to 10 young rats. The basal ration was modified by replacing corn starch with glycerol and propylene glycol, respectively, at levels of 1, 3, 6, 10, 15, 20, 30, 40, 50, and 60 parts per 100 of total ration. Consequently, the first series of experiments comprised 21 dietary groups.

In a second series of experiments, groups of young rats were fed 4 basal diets identical with that of the first series with the exception that in 3 of these starch was replaced by glucose, sucrose, or dextrin, respectively. In this series, propylene glycol and glycerol were fed only at a 30% level, replacing, in each case, 30 parts of the carbohydrate.

A third series of feeding trials was conducted in which propylene glycol and glycerol were fed in isocaloric amounts at a level approximating 30% of the diet.

In the first series of experiments, no marked differences in growth response could be detected until the alcohols were fed at the 30% level. At this level the glycerol-fed animals grew at an approximately normal rate, while the propylene glycol animals grew less rapidly. Young rats, starting at 21 to 28 days of age, lost weight and died in a few days when the diet contained 40, 50, or 60% of propylene glycol. When young rats received diets containing comparable amounts of glycerol, growth was depressed and some animals eventually died but the majority of the animals survived the experimental period. Older (half grown) animals were able to tolerate the higher levels of propylene glycol but their growth responses were never equal to those of the animals receiving comparable amounts of glycerol.

When rats received propylene glycol and glycerol diets during alternate weeks (at the 30% level), growth responses were generally increased during glycerol periods and depressed during propylene glycol periods. While glycerol diets were consumed more rapidly because of palatability, calculations show that the glycerol-fed rats made greater gains per caloric unit of food intake. No differences could be noted when other carbohydrates replaced corn starch in the basal ration. Livers from glycerol-fed rats were invariably richer in glycogen than livers from rats receiving comparable amounts of propylene glycol.

A series of metabolism studies was also conducted in which groups of half-grown rats were fed comparable diets containing increasing amounts of glycerol or propylene glycol during alternate weeks. Without exception, rats excreted in their urine the greatest percentage of the ingested water, the greatest percentage of the thiamine intake, and the greatest percentage of the polyhydric alcohol intake during the weeks in which the propylene glycol-containing diets were fed. At the highest level of intake, more than 25% of the ingested propylene glycol was accounted for in the urine while in no instance was more than 1.5% of the ingested glycerol accounted for. This finding may partially explain an earlier observation that glycerol-fed rats made the greatest body weight gains per caloric unit of food intake.

While those groups of rats receiving the glycerol-containing diets gave birth to a greater number of litters and a greater number of young per litter, they were also more successful in weaning a higher percentage of the young than were similar groups of rats subsisting on comparable propylene glycol-containing diets. Reproduction and subsequent rearing of young were abnormally low with all groups of animals, including those on the purified basal ration. An attempt was made to correct this situation by addition of vitamin and mineral supplements without success. Consequently, further attempts to continue reproduction studies with purified diets were abandoned.

Reproduction studies with diets composed of the breeding colony diet (70%) and glycerol (30%) or propylene glycol (30%) resulted in some very significant differences. Rats which have received the glycerol-containing diet are now in the fifth generation, while second generation young have not yet been successfully weaned by the propylene glycol fed mothers. A more extensive series of diets which contain proteins, minerals, and vitamins from natural food sources and in which part of the energy is furnished as glycerol or as propylene glycol is now under investigation. A report on this phase of the investigation, together with the details of the studies herein reported, including pathological findings, will be forthcoming.

The concentrations of glycerol and propylene glycol in some of these diets were much greater than concentrations ordinarily ingested, but the information obtained may have a pertinent bearing on the relative toxicity of the two polyhydric alcohols.

14724

Comparative Effectiveness of Calcium and Strontium in Plasma Coagulation.

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The classic work of Arthus and Pagés[1] and Ringer and Sainsbury[2] established the importance of calcium for blood coagulation. These investigators also tried to replace this ion by strontium. They found that this element is less effective in producing blood clotting than calcium. Their experiments were of a qualitative nature, and the subject was later studied under more quantitative conditions by Mallenby,[3] using bird plasma, and by Heard,[4] using oxalated cat blood. Both found that the coagulation time with strontium was considerably longer than with equivalent amounts of calcium. Woehler[5] has reviewed further work on this subject. It appears, however, that no comparative study has been undertaken on human blood plasma with the more quantitative methods developed in recent years.

Methods. The following experiments were conducted on the oxalated plasma of seven

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[1] Arthus, M., and Pagés, C., *Arch. de Physiol.*, 1890, **12**, 739.

[2] Ringer, S., and Sainsbury, H., *J. Physiol.*, 1890, **11**, 369.

[3] Mallenby, J., *J. Physiol.*, 1909, **38**, 28.

[4] Heard, W. N., *J. Physiol.*, 1917, **51**, 294.

[5] Woehler, E., *Erg. d. Physiol.*, 1929, **28**, 443.

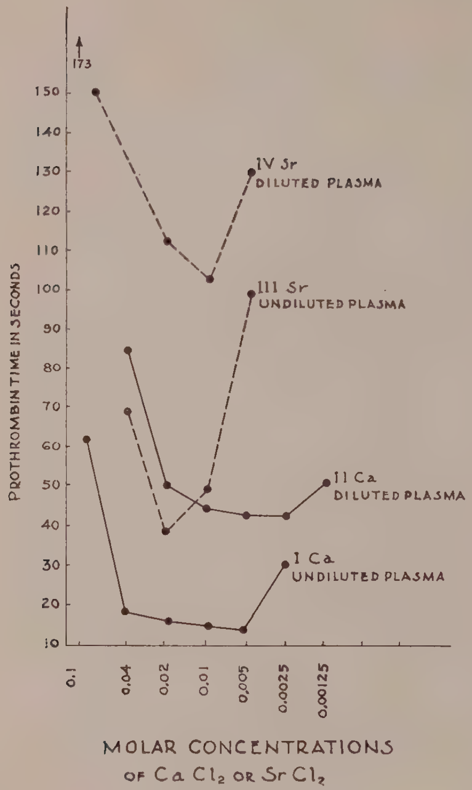

FIG. 1.

healthy humans using the method of Quick[6] for the determination of the prothrombin times. Both undiluted plasma and diluted plasma in a concentration of 12.5% were used, since it is known that diluted plasma tends to magnify minor changes in prothrombin time which are not as evident in the undiluted plasma. For the determination 0.1 cc of plasma was mixed with 0.1 cc of a suspension of thromboplastin prepared from rabbit brain by acetone desiccation. To this mixture, maintained at 37.5°C in a water bath, 0.1 cc of calcium chloride or strontium chloride in various molar concentrations was added. The mixture was continually stirred with a nichrome wire until clotting occurred.

Results and Discussion. The data summarized in Fig. 1 show the curves constructed from the averages of the determinations. The effect of varying the calcium concentration upon the prothrombin time of concentrated chicken plasma has been studied by Quick.[7] He found the shortest prothrombin time with the addition of a 0.005 and 0.0025 molar $CaCl_2$. Pohle and Stewart[8] conducted similar studies with undiluted human plasma. They found a prothrombin time of 11 seconds with 0.01 molar $CaCl_2$. The shortest prothrombin time was 10 seconds with 0.005 molar $CaCl_2$, while with 0.0025 molar $CaCl_2$ the prothrombin time was again slightly longer, 10.5 seconds. The prothrombin time became markedly prolonged with higher or lower concentrations. Prothrombin times in our experiments were slightly longer at the corresponding calcium levels, which was probably due to the use of a somewhat less potent thromboplastin. Our shortest prothrombin time occurred with 0.005 molar $CaCl_2$ concentration, the same as given by Pohle and Stewart.[8] However, the slope of our curve in the lower concentration range is steeper. For instance, the prothrombin time is already 30 seconds with 0.0025 molar $CaCl_2$. Apparently the effect of different $CaCl_2$ concentrations upon the prothrombin time of diluted plasma has not been reported. The curve constructed from the values obtained with 12.5% plasma is similar in shape to the one of the concentrated plasma, but the concentration range in which the clotting time shows but slight change is shifted to the lower calcium concentrations.

If we compare these findings with the data obtained with $SrCl_2$, we notice that the prothrombin times are considerably longer than those with $CaCl_2$, and that the optimal $SrCl_2$ concentration is much higher for both the undiluted and the diluted plasma. These optimal concentrations of $SrCl_2$ are 0.02 molar for whole plasma and 0.01 molar for diluted plasma. The range of concentrations in which the prothrombin times show but slight change is much smaller for strontium than for calcium, the curve rising sharply on either side of this zone. This is interpreted as a further indication of the greater specificity of calcium as compared with strontium for plasma coagulation.

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[6] Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis,* Charles C. Thomas, 1942.

[7] Quick, A. J., *Am. J. Physiol.,* 1940, **131**, 455.

[8] Pohle, F. J., and Stewart, J. K., *Am. J. Med. Sci.,* 1939, **198**, 622.

We have conducted a number of additional studies using 12.5% rat plasma and the method of prothrombin determination as modified by Link and collaborators.[9] Due to the fact that individual rats vary from each other in their normal prothrombin time more than the humans do, the results are not as conclusive. However, our findings indicate that here also the prothrombin times with $SrCl_2$ were considerably longer than with $CaCl_2$. As in human plasma, no marked changes in prothrombin time occurred with $CaCl_2$ between 0.02 and 0.005 molar, while with $SrCl_2$ the optimal concentration was at 0.01 molar with a rather steep rise of the prothrombin times with either 0.02 or 0.005 molar $SrCl_2$. It was also observed with the plasma of both humans and rats that the clot formed with $SrCl_2$ was not as firm as with $CaCl_2$, and this was the more evident the farther away the $SrCl_2$ concentration was removed from the optimum.

Summary. The comparative effectiveness of $CaCl_2$ and $SrCl_2$ as clotting agents in various concentrations was studied with undiluted and diluted human plasma. It was found that the prothrombin time with $SrCl_2$ is longer than with $CaCl_2$, and also that the optimal molar concentration of strontium is much higher. Further, the range of concentrations with which a relatively short prothrombin time can be obtained is much smaller with $SrCl_2$ than with $CaCl_2$. This is taken as further evidence for the greater specificity of the calcium ion. Corresponding experiments with diluted rat plasma gave essentially similar results.

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[9] Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

14725 P

Efficacy of M-Amino Ethyl Benzoate as an Anesthetic for Amphibian Embryos.

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From Washington Square College of Arts and Sciences, New York University.

Chloretone, cocaine, novocaine, and freezing have been used as anesthetics for living embryos but have not proven to be entirely satisfactory. Rothlin[1] described a meta-aminobenzoic acid-ethylester in the form of methansulphonate (isomeric with anesthesin) which is highly soluble in water, 3 times less toxic than novocaine and 10 times less toxic than cocaine. This anesthetic had been used by Sandoz and Stryzowski,[2] Witschi,[3] Christensen,[4] Rotmann,[5] Glucksohn,[6] Goodrich and Nichols,[7], Baudin,[8] and Sato,[9] but these investigators were interested in it only as a tool.

This anesthetic is commercially known as M.S.222.* It has no effect on ciliary action or on the process of differentiation. Both cilia and muscle will develop in an embryo while under the influence of this anesthetic but the cilia alone will function. This not

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[1] Rothlen, E., *Schweiz Med. Wochr.*, 1932, **45**, 1042.

[2] Sandoz, M., and Stryzowski, R., *Bull. Soc., Vand. Sc. Nat.*, 1920, **53**, 199.

[3] Witschi, E., *J. Exp. Zool.*, 1927, **47**, 269.

[4] Christensen, K., *Anat. Rec.*, 1931, **48**, 241.

[5] Rotmann, E., *Roux Arch. f. Entwicklungsmechanik*, 1931, **124**, 747.

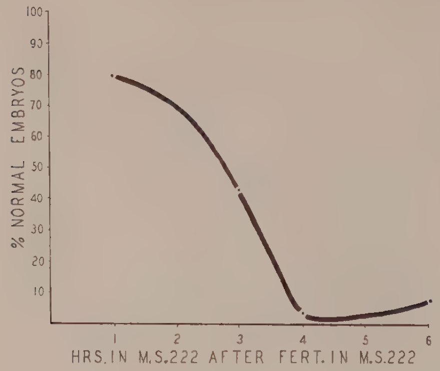
[6] Glucksohn, S., *Roux Arch. f. Entwicklungsmechanik*, 1931, **125**, 341.

[7] Goodrich, H. B., and Nichols, R., *Anat. Rec.*, 1931, **51**, 513.

[8] Baudin, L., *C. R. Soc. de biol.*, 1932, **110**, 235.

[9] Sato, T., *Roux Arch. f. Entwicklungsmechanik*, 1932, **122**, 451.

* This anesthetic is available through M. Sandoz & Co., New York City.



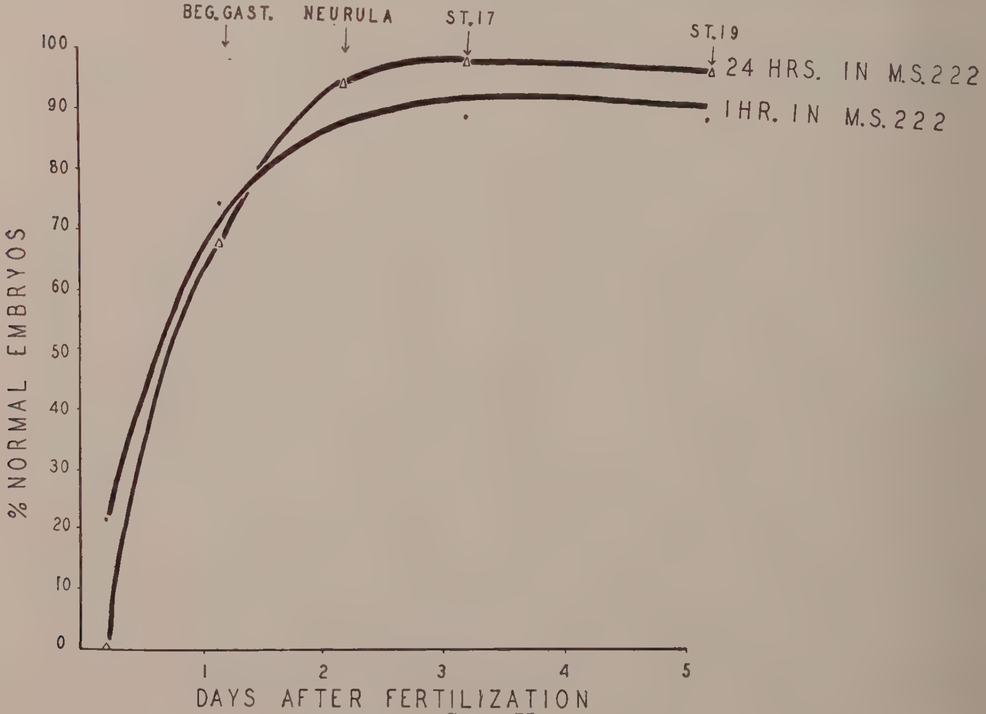
Graph I.

only brings out the major difference between ciliary and muscular action but also the independence of morphological and functional differentiation, in the case of muscle.

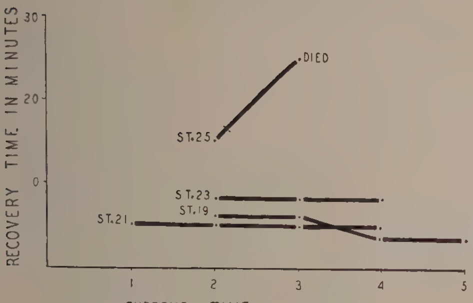
The purpose of this paper is to report some experiments which were designed to determine (1) any effect of M.S.222 on fertilization and early cleavage, (2) the speed of anesthetization at different stages of development, (3) the speed of recovery from complete anesthesia, (4) the effect on embryonic development of prolonged exposure to the anesthetic, and (5) the critically sensitive periods in early embryonic development following brief exposure to the anesthetic.

The dilution of M.S.222 used was 1/3,000 in spring water. Ovulation in the frog, *Rana pipiens,* was induced by pituitary injection and controls were kept for all experiments. Each point on Graphs I and II represents averages of 50 animals while points on Graphs III and IV represent averages of 5 readings. All experiments have been duplicated.

Graph I represents the data from fertilization of eggs in the anesthetic. A single testis was taken from each of 6 male frogs and all of them minced in 20 cc of 1/3,000 M.S.222 in spring water. The other 6 testes were minced in the same volume of pure spring water, as control material. The uterine eggs were then stripped into the sperm suspensions and were subsequently flooded with the same solution, to allow rotation. In this graph it



Graph II.



Graph III.

must be pointed out that the control eggs showed only 80% development so that this level must be considered maximum and normal. It appears that M.S.222 does not interfere with fertilization but that with increasing exposure to the anesthetic, prior to removal to spring water, there is a decrease in the percentage of normal development to a minimum at the 4-hour level. All of these points include the period of drastic changes that are presumed to occur in the egg immediately after fertilization.

In Graph II is found a comparison of the effect of 1-hour and 24-hour exposure to the anesthetic at different stages of development. All eggs were from a single female and were fertilized in spring water in which the controls showed 100% normal development. The two curves are much the same, indicating that the deleterious effects are more manifest when the exposure to the anesthetic is made prior to gastrulation (whether for 1 or 24 hours); slightly less so before neurulation; and negligible after neurulation. That is, after the embryo has passed through the critical periods of gastrulation and neurulation, even the 24-hour exposures were relatively harmless.

In Graph III is shown the results of exposure of later stage embryos for periods of 1 to 5 hours of anesthetization. It will be noted that stages 19, 21, and 23[10] showed recovery in 5 to 10 minutes no matter how long the exposure to M.S.222 while stage 25 died after 3 hours exposure. This exception is probably related to the fact that at this stage the operculum opens and there is a shift from external to internal gill respiration. These embryos exhibit a typical oedema and rupture of the cephalic blood vessels.

In Graph IV is shown the rate of recovery of the 4 stages of frog embryo development

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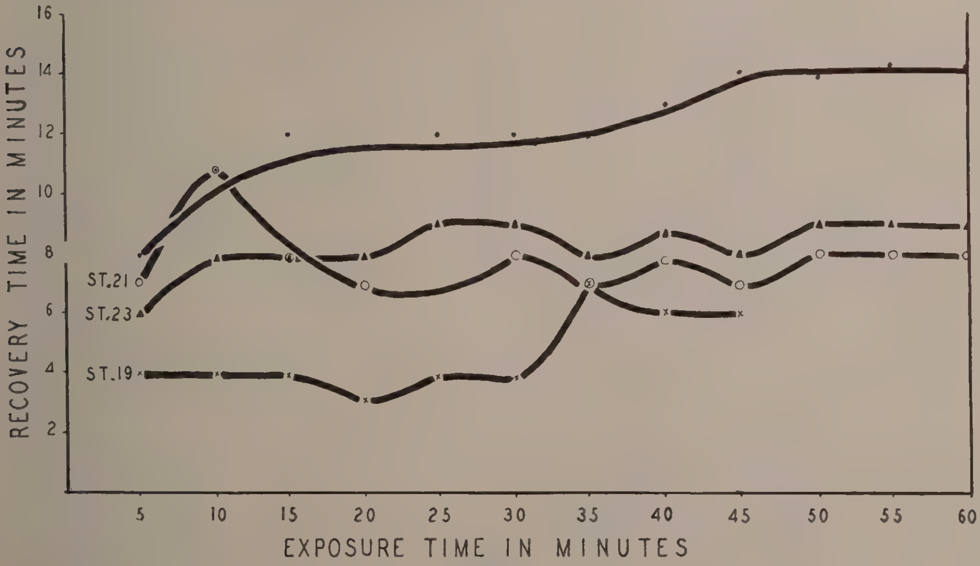
[10] Rugh, R., *Experimental Embryology*, 1943, N. Y. U. Press.



M.S.222
Graph IV.

(stages 19, 21, 23, and 25) following various exposure periods from 5 minutes to 1 hour. The time lapse for complete muscular immobilization varied from 30 to 80 seconds, and the recovery time ranged from 4 to 14 minutes, depending upon the duration of anesthetization and the stage of development considered. Here too it will be noted that recovery time consistently was increased only in the case of stage 25.

Summary and Conclusions. (1) The anesthetic M.S.222 in 1/3,000 concentration in spring water has no effect on the motility or the fertilizing power of frog spermatozoa. (2) Frog's eggs fertilized in this anesthetic and allowed to remain for 1 hour will develop as well as do the controls. Exposure for longer periods will show an increasing percentage of abnormal embryos. (3) Embryos that show muscular activity (stage 19 and older) are completely immobilized (except for ciliary activity) within 80 seconds at ordinary laboratory temperatures, some embryos becoming quiet within 30 seconds. Recovery rate (*i.e.*, the return to muscular activity) ranges from 4 to 14 minutes, depending more on the stage of development than on the duration of anesthetization. (4) Prolonged exposure to the anesthetic produces abnormal embryos if the exposure is made prior to neurulation and kills the embryos at stage 25. This latter instance may be due to the change in the respiratory mechanism at that time. (Urodele larvæ do not show this reaction, and may be anesthetized at least up until metamorphosis.) (5) The anesthetic has no effect on ciliary action but is quick in its effect on muscular activity; it is non-toxic in the dilution of 1/3,000 in spring water for periods adequate for surgical operations; and recovery time is uniformly brief. M.S.222 has proven to be a very satisfactory anesthetic for amphibian embryos.

14726

Effect of Starvation and Ageing on the Acid-Soluble Phosphate Components of the Liver.

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In contrast to the abundance of information on muscle phosphates, comparatively little is known regarding the acid-soluble phosphates of the liver. In connection with a tracer study of the phosphate metabolism of the liver with P^{32}, a comparison was carried out of the distribution of the acid-soluble phosphate fractions of the liver of the rat under certain extremely varied conditions of life. The analytical procedures are described elsewhere.[1]

Effect of Starvation. The distribution of the different phosphorus fractions in the livers of rats fasted for 12, 24, 48, 72, and 144 hours are recorded in Table I. For purposes of comparison, the table contains the phosphorus partition in the liver of rats who had access to food until they were sacrificed. The results in Table I, in general, are in agreement with the findings of Rapoport, Leva, and Guest.[2]

Effect of Age. That the acid-soluble phosphates of the liver changed with age was observed by Kay.[3] We considered it of value to investigate the nature of these changes more thoroughly.

For the purposes of this study, several litters of stock rats were sacrificed at different ages, beginning with 21-day-old (several days prior to weaning) and the phosphate content of the various acid-soluble fractions determined The results are given in Table II.

From Table II it will be noted that the total

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[1] Kaplan, N. O., and Greenberg, D. M., *J. Biol. Chem.*, in press.

[2] Rapoport, S., Leva, E., and Guest, G. M., *J. Biol. Chem.*, 1943, **149**, 57, 65.

[3] Kay, H. D., *J. Biol. Chem.*, 1932, **99**, 85.

TABLE I.

Effect of Time of Fasting on Distribution of Acid-Soluble Phosphate Fractions of Rat Liver.*

		Barium insoluble					Barium soluble		
Hrs of fast	Animals No.	Total acid soluble P	Inorganic P	Labile ATP P	Non-labile ATP P	Residual† P	Hg‡ ppt P	Pb§ ppt P	Alcohol‖ ppt P
0	10	100.8 ±1.6	21.2 ±0.37	15.6 ±0.45	7.8	9.6 ±0.29	11.6 ±0.32	7.9 ±0.26	30.2 ±0.88
12	12	95.1 ±1.3	24.0 ±0.45	12.0 ±0.32	6.0	9.0 ±0.37	10.6 ±0.70	7.1 ±0.55	27.7 ±0.80
24	5	91.1 ±1.7	26.3 ±0.60	10.2 ±0.47	5.1	8.5 ±0.40	10.8 ±0.60	7.2 ±0.48	25.0 ±0.75
48	4	89.0 ±1.6	26.2 ±0.72	8.4 ±0.44	4.2	9.0 ±0.73	10.5 ±0.56	6.8 ±0.39	24.0 ±0.97
72	6	83.9 ±2.6	27.7 ±0.94	7.4 ±0.27	3.7	8.0 ±0.33	9.9 ±0.33	7.2 ±0.42	21.3 ±0.88
144	4	81.5 ±2.1	29.0 ±0.75	7.2 ±0.61	3.6	8.4 ±0.58	11.4 ±0.62	6.0 ±0.50	20.7 ±0.86

* All values are in mg P per 100 g fresh liver. Measure of variability is standard error of the mean.

† This fraction consists largely of phosphoglycemic acid.

‡ This fraction contains nucleotides other than adenosine triphosphate.

§ This fraction mainly contains inorganic phosphate that has escaped previous precipitation.

‖ This fraction consists largely of glycerol phosphate but it also contains some hexose monophosphates.

TABLE II.

Influence of Age on Liver Acid Soluble Phosphates.*

			Barium insoluble					Barium soluble		
Age of rat in days	Wt of rat in g		Total acid soluble P	Inorganic P	Labile P in ATP	Non-labile P in ATP	Residual P	Hg Ppt P	Pb Ppt P	Alcohol Ppt P
18	35†	a	85.5	31.8	7.2	3.6	17.2	11.3	4.7	18.9
19	42†	b	82.0	28.2	7.5	3.8	13.3	11.8	5.5	17.9
23	55	c	88.4	27.0	9.1	4.6	14.2	11.7	5.8	20.8
23	68	b	92.2	27.6	7.2	3.6	12.4	10.2	6.0	22.8
30	79	c	87.0	26.2	10.2	5.1	13.2	10.4		17.5
32	82	a	91.1	27.7	9.8	4.9	11.7	10.0	7.0	21.2
40	112	c	92.4	26.1	9.2	4.6	10.8	9.9	8.3	22.8
44	130	b	94.1	28.7	9.4	4.7	9.8	9.5	5.5	24.4
45	96	a	95.5	26.4	11.3	5.7	9.8	8.5	6.7	23.3
50	138	c	97.5	23.8	11.8	5.9	7.8	11.7	6.7	25.9
60	152	a	91.2	24.8	11.4	5.7	8.7	10.5	5.1	27.8
60	174	b	100.0	26.3	11.9	6.0	9.1	10.5	9.1	26.8
65	204	c	95.9	22.8	13.6	6.8	8.1	11.3	5.9	28.5
75	225	a	93.0	24.4	13.0	6.5	7.4	9.2	6.8	26.0
75	275	b	98.1	25.2	13.9	7.0	9.3	9.7	6.0	27.8
100	319	b	90.9	23.3	11.4	5.7	8.5	9.0	7.4	26.7
180+	402		82.6	22.4	10.5	5.3	7.9	8.5	5.5	24.4
180+	510		78.5	22.5	9.1	4.6	7.3	8.9	6.1	22.0

* All values are in milligrams per 100 g fresh liver. For significance of the different fractions see [1] and Table I.

† Unweaned rats.

All animals were fasted for a period of 12 hours before sacrificing. Rats listed with same letter are litter mates.

acid-soluble phosphate increases up to maturity and then drops off again at an advanced age. The inorganic phosphate decreases progressively with advancing age. The change in the ATP of the liver parallels that of the total phosphate. The decrease in the total acid-soluble and ATP phosphate in the 400 to 500 g rats may be due to an increase in the fat content of the liver in these animals.

It is of interest that the livers of the younger animals contain a higher content of phosphorus in the barium residual fraction and a lower content in the alcohol precipitate than do the old ones. With increasing age, there is a shift of phosphorus from the barium residual fraction to the alcohol precipitate.

No definite conclusions can be drawn regarding the significance of the phosphate changes which occur with age, until more information is available on the relationship of age to the metabolic activities of the liver.

Summary. Fasting causes a decrease in the total acid-soluble phosphate, adenosine triphosphate, and the glycerol phosphate fractions of the liver. With advancing age there is a shift of phosphate from the fraction containing phosphoglyceric acid to the fraction containing glycerol phosphate.

14727 P

Relation of Riboflavin Deficiency to Spontaneous Epidemics of *Salmonella* in Mice.

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In a previous publication[1] it was reported that vitamin A-deficient mice and rats are highly susceptible to a *Salmonella* infection. However, it was found that this increased susceptibility appeared only in the late stages of avitaminosis and that animals given adequate amounts of the vitamin, but the same caloric intake as those on the vitamin-free diet were equally susceptible. In this type of infection vitamin A was not the "anti-infection vitamin;" the same decreased resistance resulted from a reduced food intake.

Below we present results on the effect of riboflavin-deficient diets on spontaneous *Salmonella* epidemics in mice. Our stock mice became infected with *Salmonella* and occasionally single mice died from the infection without any serious epidemic outbreaks. Young mice, four weeks old, were divided into 3 groups. (1) Control group, received the following diet *ad libitum*: Casein (alcohol extracted), 15%; rice flour, 71%; olive oil, 10%; salt mixture, 4%. Each mouse received in addition 50 I.U. vitamin A, 2 I.U. vitamin D twice a week, and 0.1 cc of a yeast extract daily. The yeast extract was irradiated in alkaline solution with a 200-watt lamp at a distance of 25 cm for 8 hours, then neutralized and 25 γ thiamin, 25 γ calcium pantothenate, and 25 γ riboflavin per ml added. (2) Riboflavin-deficient group, received the same diet as the controls, without riboflavin. (3) Paired-fed group, received the same diet as the control except that the daily amount of the basic ration was weighed out and equalled that eaten the previous day by the animals on the riboflavin-deficient diet.

Eight series of experiments have been carried out with consistent results. In general they fall into 3 main types.

In the first series mice from the mixed stock were divided into groups of 15, of approximately the same average weight. The animals were kept under observation 21-28 days during which mice which died were cultured. At the end of that period the remaining mice were killed and the liver, spleen, and mesentery gland cultured for *Salmonella*.

In the second series the mice were divided into groups of litter-mates. Each litter was

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* Dr. I. J. Kligler died September 24, 1944.

[1] Kligler, I. J., Guggenheim, K., and Henig, E., *J. Hyg.*, in press.

TABLE I.
Relative Susceptibility of Mice Kept on Full, Riboflavin Deficient, and Isocaloric Diets to a Spontaneous Infection with *Salmonella*.

Exp.	Source of mice	Observation time before culturing	No. of mice	No.* died	No. alive and cultured	Total infected	Liver pos.	Spleen pos.	Spleen and liver pos.	Mesentery gland positive
I.										
Rib. Def.	Mixed stock	3-4 weeks	78	27	51	42	13	11	8	40
P.F.			69	17	52	26	4	5	4	26
Control			30	4	26	5	2	2	0	5
II.										
Rib. Def.	Litter mates	3-4 weeks	30	3	27	23	14	16	13	22
P.F.			30	1	29	20	11	13	11	20
Control			30	0	30	13	3	3	1	13
III.										
Rib. Def.	Litter mates	2 weeks	42	0	42	27	10	10	9	27
P.F.			42	0	42	20	8	7	7	20
Control			42	0	42	12	1	2	1	12

* Of the 52 mice which died it was possible to culture 39; all of them showed a general infection of *Salmonella*.

taken directly from the mothers and divided into 3 groups. The object was to obtain as nearly as possible a uniform distribution of initially-infected mice in each group. In all other respects the procedure was the same as in the first series.

The third series also consisted of littermates, but the mice were killed after 14-15 days instead of 3-4 weeks. In order to assure infection one mouse infected *per os* with 0.05 cc culture of *Sal. typhimurium* was introduced into each cage of 15, three days after the infection. The purpose of this series was to see whether different degrees of susceptibility existed in the early stages of avitaminosis, *i.e.*, after 2 weeks on the deficient diet.

The results are summarized in Table I. It is evident that the riboflavin-deficient mice had a markedly higher rate of infection than the controls; the fatality in the experiments of long duration was nearly 3 times as great and the liver and spleen infections in all survivors about 3-4 times as high. The paired-fed animals fared better consistently than the deficient ones, but came a close second to them. The riboflavin-deficient animals differ from those receiving a vitamin A-deficient diet in that in the former loss of appetite sets in promptly after the first week and the low level of food intake remains constant throughout the period of observation, while in the latter the food intake remains almost normal for 2 or 3 weeks and only then drops sharply. It would seem that riboflavin deficiency acts more critically to the organism than vitamin A deficiency and causes a sharp loss of resistance within two weeks; in this case too, however, starvation as distinct from avitaminosis is also a determining factor in enhanced susceptibility. These findings are consistent with those of Wooley and Sebrell[2] with experimental nasal infections with pneumococci in riboflavin-deficient mice.

Summary. Controlled nutrition experiments with mice kept on a riboflavin-deficient diet show that these animals are much more highly susceptible to a spontaneous *Salmonella* infection than are control mice from the same stock and mothers. Isocaloric paired-fed controls given a full vitamin ration show an intermediate degree of susceptibility between the riboflavin-deficient and the controls.

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[2] Wooley, J. G., and Sebrell, W. H., *Pub. Health Rep.*, 1942, **57**, 149.

14728 P

Connective Tissue Neoplasms Produced in Dogs by Methylcholanthrene.

R. M. Mulligan.

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Passey[1] obtained cutaneous melanomas in 2 mongrel Airedale bitches by painting their skin with tar. Hueper, Wiley, and Wolfe[2] found carcinomas of the urinary bladder in 3 mongrel female dogs following the subcutaneous and oral administration of commercial beta-naphthylamine. Bonser[3] fed pure beta-naphthylamine to 3 mongrel male Airedales which developed carcinomas of the urinary bladder.

In some recent experiments, in which methylcholanthrene was administered under nembutal anesthesia to dogs, a male white Scotch terrier mongrel (No. 53) about 3 years old and weighing 9.5 kg was injected (3/25/42) subfascially in the left flank with 50 mg of this carcinogen dissolved at about 45°C in 3 cc each of benzene and paraffin (M.P. 51.6°C). At the end of 297 days a nodular tumor in direct continuity with the injected material was found in the left lower quadrant of the ventral abdominal wall, where on the 327th day autopsy disclosed a neoplasm consisting of 6 nodules set close together, ranging from 10 to 22 mm in their greatest diameters, varying from white to dark red and white streaked to dark red in color, and firm to moderately firm in consistency. With hematoxylin and eosin stain the darker areas in the nodules were partly necrotic and suffused with recent hemorrhage. The lighter areas contained the best preserved parts of the neoplasm (Fig. 1), the constituent cells of which were spindle-shaped, possessed plasmodial pale acidophilic processes, and had indistinct borders so that they overlapped in syncytial fashion. The finely chromatinized diffusely vesicular nuclei of the cells were elongated or oval, varied greatly in size, and contained 1 to 4 basophilic nucleoli each. Many tumor cells possessed 2 to 5 nuclei. A few mitotic figures were present. Some fine acidophilic intercellular fibrillæ were basophilic with Mallory's connective tissue stain, indicating their collagenous nature. The diagnosis was connective tissue neoplasm with the histologic characteristics of an immature fibrosarcoma. No metastases were observed.

Another dog, a male black water spaniel mongrel (No. 52) about 1½ years old and weighing 8 kg, was injected (3/17/42) subfascially in the left flank with 40 mg of methylcholanthrene dissolved at about 45°C in 3 cc each of benzene and paraffin. On the 361st day, 100 mg of methylcholanthrene in a gelatin capsule were implanted subfascially through a small skin incision in the right side of the thorax over the 6th and 7th ribs. On the 735th day a nodule in direct continuity with the first methylcholanthrene given was found in the left lower quadrant of the ventral abdominal wall, where necropsy on the 791st day revealed a 23 mm firm, white, bulging neoplasm along the lateral border of the left rectus muscle. With hematoxylin and eosin stain the constituent cell was spindle-shaped. possessed tapering acidophilic processes, and had fairly distinct borders. The finely chromatinized nuclei of the cells were oval or oblong and contained 1 to 3 basophilic nucleoli each. Many mitotic figures (Fig. 2) were present. Some tumor cells showed 2 to 4 nuclei. The stroma contained pale basophilic material and toward the center of the tumor were areas of edema. Many capillaries were found. Numerous groups of acidophilic intercellular fibrillæ were basophilic with Mallory's connective tissue stain. The diagnosis was connective tissue neoplasm with the histologic characteristics of a mature fibrosarcoma. No metastases were observed.

The second methylcholanthrene implant revealed a 1.5 mm nodule of the carcinogen, but no neoplasm. This dog, the third dog

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[1] Passey, R. D., *J. Path. and Bact.*, 1938, **47**, 349.

[2] Hueper, W. C., Wiley, F. H., and Wolfe, H. D., *J. Indust. Hyg. and Toxicol.*, 1938, **20**, 46.

[3] Bonser, G. M., *J. Path. and Bact.*, 1943, **55**, 1.

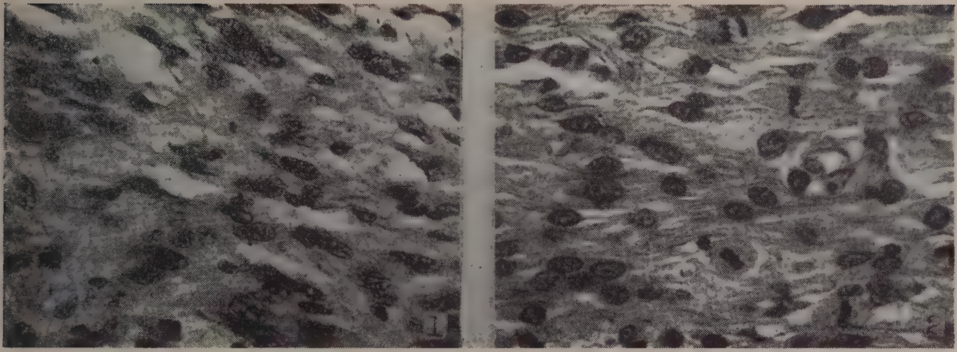

Fig. 1. ×335. Fig. 2. × 450.
Connective Tissue Neoplasms Produced by Methylcholanthrene. Description in text.

described in a recent communication,[4] was fed stilbestrol for the 280 days between the 34th and 314th days of the methylcholanthrene medication. On this 314th day, 500 mg of stilbestrol were implanted subfascially in the right upper quadrant of the ventral abdominal wall. The subsequent events and the clinical recovery of this animal have been reported.[5]

Methylcholanthrene was also administered in doses of 30 to 100 mg to 6 other mongrel dogs, 3 males (the 1st, 2nd, and 4th dogs previously described[4]) and 3 females, about 1½ to 3 years old and weighing 7 to 14 kg at the start of the experiments lasting 150 to 618 days, but without the production of neoplasms demonstrable at autopsy at or near the site of injection or in other locations.

Summary. Connective tissue neoplasms with the histologic characteristics of fibrosarcoma were produced in 2 dogs by the subfascial injection of methylcholanthrene.

Note: Dog 53 was presented at the March 5, 1943, meeting and dog 52 at the May 26, 1944, meeting of the Rocky Mountain Section of the Society for Experimental Biology and Medicine.

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[4] Mulligan, R. M., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 21.

[5] Mulligan, R. M., *Am. J. Path.*, 1944, **20**, 865.

14729

Effects of Fourneau 883 and Fourneau 933 on Late Renal Hypertension in the Rat.

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In a recent study of the effects of nembutal and yohimbine on the blood pressure of normal and hypertensive rats, Reed *et al.*[1] have shown that the blood pressure of *late* hypertensive animals is lowered by nembutal anesthesia and sympatholytic-adrenolytic doses of yohimbine, whereas the blood pressure of *normal* or *early* hypertensive animals is unaffected by these substances. These data were interpreted to signify a change in the mechanism of maintenance of renal hypertension in the animals used, the initial renal humoral disturbance which precipitates hypertension being superseded by neurovascular or other alterations which maintain blood pressure after the dis-

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[1] Reed, Racheal K., Sapirstein, Leo A., Southard, F. D., Jr., and Ogden, Eric, *Am. J. Physiol.*, 1944, **141**, 707.

TABLE I.
Effects of F 883 and F 933 on Systolic Blood Pressure of Normal and Late Hypertensive Rats.

Description.	No.	Systolic pressure Before inj.	After inj.	Diff.
F 883 (5 mg per rat).				
Late Hypertensive	16	165	126	—39
Normal	8	117	115	— 2
F 933 (10 mg per rat).				
Late Hypertensive	16	151	144	— 7
Normal	8	121	119	— 2

appearance of the original humoral imbalance.

The effects of the sympatholytic-adrenolytic drug yohimbine on late hypertension suggested that this state was maintained either through the sympathetic nervous system as a whole or through nervous involvement of the adrenal medulla alone. In the present investigation we have studied the effects of a purely adrenolytic substance, piperidino-methyl-benzo-dioxane (Fourneau 933) and a sympatholytic and adrenolytic drug, diethyl-amino-methyl-benzo-dioxane (Fourneau 883) on the blood pressures of late hypertensive animals. Evidence that in the doses used F933 is purely adrenolytic and that F883 has both adrenolytic and sympatholytic effects has been presented by Bacq and Fredericq[2,3] and others.

Methods. All hypertensive rats had been hypertensive (systolic pressure $>$ 150) for at least 3 months before the effects of the 2 drugs were studied. Hypertension was induced by partial ligation of one renal artery according to the method of Wilson and Byrom.[4] Blood pressures were taken on unanesthetized animals by the tail plethysmograph method of Williams, Harrison, and Grollman.[5] Both substances were used on each hypertensive rat, an interval of one week being allowed after injection of the first before the effect of the second was determined. Normal controls were from the same stock and were of the same age and weight as the hypertensives. The drugs were made up just before injection in solutions containing 5 or 10 mg per cc. All injections were made intraperitoneally. Blood pressures were recorded immediately before and 30-45 minutes after the injection.

Findings. Fourneau 883 (sympatholytic and adrenolytic) at a dosage of 5 mg per rat lowered the blood pressure of late hypertensive rats to nearly normal levels. The maximum effect was observed in 30-120 minutes, and some effect persisted for 24 hours. Fourneau 883 was without effect on the blood pressure of normal animals.

Fourneau 933 (adrenolytic), at a dosage of 10 mg per rat, was without effect on the blood pressure of either hypertensive or normal rats.

The results are summarized in Table I.

Discussion. The lowering of blood pressure of late hypertensives by the sympatholytic and adrenolytic drugs yohimbine[1] and F883, and the failure of the adrenolytic drug F933 to exert any effect on the blood pressure of late hypertensive animals indicate that sympathetic activity rather than hyperfunction of the adrenal medulla is at fault in the mechanism which follows the renal mechanism in maintaining hypertension in the rat.

Summary and Conclusions. 1. The sympatholytic-adrenolytic drug F883 lowered the blood pressure of 16 late hypertensive rats to a nearly normal level. The blood pressure of normal rats was unaffected. 2. The adrenolytic drug F933 failed to lower the blood pressure of either late hypertensive or normal rats. 3. It is concluded that secondary hypertension of renal origin is mediated through the sympathetic nervous system rather than through the adrenals.

Our thanks are due to Dr. Eric Ogden for suggesting this experiment, and to Dr. George Emerson for supplying the two dioxane derivatives.

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[2] Bacq, Z. M., and Fredericq, H., *C. R. Soc. de biol.*, 1934, **117**, 806.

[3] Bacq, Z. M., and Fredericq, H., *C. R. Soc. de biol.*, 1935, **118**, 183.

[4] Wilson, C., and Byrom, F. B., *Lancet*, 1939, **1**, 136.

[5] Williams, J. R., Jr., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

14730

Effect of Adrenotrophic Hormone of Pituitary Gland on Ability of Tissue to Synthesize Acetylcholine.*

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In the presence of serum[1,2] and spinal fluid[3] from patients with myasthenia gravis less acetylcholine was synthesized *in vitro* than in the presence of serum and spinal fluid from persons without myasthenia gravis. Patients with myasthenia gravis may have enlarged thymus and lymphocytic infiltration of certain tissues and are sometimes benefited by removal or irradiation of the thymus. Also, ether extracts of thymus decrease the synthesis of acetylcholine both *in vivo* and *in vitro*.[4]

It is known that there is a reciprocal relationship between the size of the adrenal cortex and thymus.[5–11] Thymic atrophy[11–17] and decrease in the amount of lymphoid tissue[16–18] following administration of the adrenotrophic hormone of the pituitary gland has also been reported. In the following experiments an attempt was made to ascertain whether injection of the adrenotrophic hormone of the pituitary gland into living animals modifies the synthesis of acetylcholine.

Experimental. The adrenotrophic hormone of the pituitary gland was prepared according to the method of Sayers, White, and Long.[19] Mice (18 to 20 g each†) were injected subcutaneously daily with 0.5 mg adrenotrophic hormone in 0.5 cc aqueous solution for 15 days. Non-injected mice and mice injected with albumin served as controls. The activity of the adrenotrophic hormone was tested by ascertaining the morphological changes induced by the hormone during the 15 days of injection (Table I). The changes observed in the thymus, adrenal glands and lymphatic tissue were similar to those found by Dougherty and White.[16]

After 15 days of injection the animals were killed by separating the head from the body. The brain was excised, 200 mg samples were weighed, finely minced and incubated for assay of the synthesis of acetylcholine. The synthesis of acetylcholine was ascertained according to the method of Quastel, Tennen-

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* This study was aided by a grant from the Josiah Macy, Jr., Foundation.

[1] Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

[2] Torda, C., and Wolff, H. G., *J. Clin. Invest.*, 1944, **23**, 649.

[3] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

[4] Torda, C., and Wolff, H. G., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 69.

[5] Hammett, F. S., *J. Metabolic Res.*, 1925, **78**, 91.

[6] Anderson, D. H., *J. Physiol.*, 1935, **85**, 162.

[7] Selye, H., *Endocrinology*, 1937, **21**, 169.

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[9] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 443.

[10] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 174.

[11] Kendall, E. C., *Ann. Rev. Biochem.*, 1941, **10**, 285.

[12] Cramer, W., and Horning, E. S., *Lancet*, 1939, **1**, 192.

[13] Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 42.

[14] Noble, R. L., and Collip, J. B., *Endocrinology*, 1941, **29**, 934.

[15] Houssay, B. A., *J. Am. Med. Assn.*, 1942, **118**, 833.

[16] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

[17] Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 135.

[18] White, A., and Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

[19] Sayers, G., White, A., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 199.

† All animals received "Big Rid" dog pellets (G. L. F. Farms, Inc., Ithaca, N.Y.), and water *ad libitum*.

TABLE I.
Effect of Adrenotrophic Hormone on Weight of Some Tissues of Mice.

Mice	No. of animals	Tissue wts as mg per 100 g of initial body weight			
		Thymus	Paired axillary nodes	Paired mesenteric nodes	Paired adrenals
Controls	45	165.0 ± 12.0	63.0 ± 2.9	135.1 ± 2.5	18.2 ± 1.6
Adrenotrophic hormone injected	25	75.9 ± 7.0	52.7 ± 3.5	95.3 ± 4.7	27.0 ± 1.3

TABLE II.
Effect of Adrenotrophic Hormone on the Ability of Brain to Synthesize Acetylcholine.

Mice	No. of animals	Amounts of acetylcholine synthesized in μ per 100 mg brain	
		Free acetylcholine	Total acetylcholine
Control	45	0.66 ± 0.024	1.48 ± 0.030
Adrenotrophic hormone injected	25	1.01 ± 0.041	2.28 ± 0.046

baum, and Wheatley[20] with minor modifications.[2] Two hundred mg minced brain, 3 cc mammalian Ringer's solution at pH 7.4, 3 mg physostigmine salicylate, and 4.8 mg glucose were shaken and incubated aerobically for 4 hours at 38°C. Because of the lability of the enzyme involved in the synthesis of acetylcholine, not more than 2 minutes were allowed to elapse between the killing of the animals and the start of shaking and incubation. After incubation the amounts of free and total acetylcholine synthesized were assayed biologically using as indicator the sensitized rectus abdominis muscle of the frog.

The amounts of acetylcholine synthesized by the brains of injected mice as compared to the controls are summarized in Table II. It was found that brains obtained from animals injected with the adrenotrophic hormone synthesized on the average 50% more acetylcholine than did brains obtained from the control animals.

Discussion. The above results indicate that injection of the adrenotrophic hormone of the pituitary gland into living animals induces not only an increase of the mass of the adrenal gland and atrophy of the thymus and the lymphatic tissue, but also causes an increase of the ability of tissue to synthesize acetylcholine.

The adrenotrophic hormone increases the secretion of the adrenal cortex.[21] The increase of acetylcholine synthesis is probably not due to an accumulation of some of the hormones of the adrenal cortex in the brain since addition of desoxycorticosterone and 4,5-dihydro-17-hydroxy-11-dehydro-corticosterone to brains of non-injected animals before incubation does not increase the amount of acetylcholine synthesized *in vitro*.[22]

The increase of the synthesis of acetylcholine may be due to an increased amount of enzyme in the brain, or to changes in the substrate for the enzyme contained in the brain. Due to the effect of the adrenotrophic hormone on metabolic processes, the brain may become a more favorable medium for the enzyme.

Since it is the opinion of the authors that the symptomatology of myasthenia gravis may be due to a decrease of the ability of nerve tissue of these patients to synthesize acetylcholine, the effect of the administration of the hormone to patients with myasthenia gravis is now being investigated.

Summary. The effect of the adrenotrophic hormone of the pituitary gland on the synthesis of acetylcholine was ascertained. Brain obtained from mice injected for 15 days with the adrenotrophic hormone synthesizes on the average 50% more acetylcholine than does brain obtained from control animals. There-

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[20] Quastel, J. H., Tennenbaum, M., and Wheatley, A. H. M., *Bioch. J.*, 1936, **30**, 1668.

[21] Sayers, G., Sayers, M. A., Lewis, H. L., and Long, C. N. H., PROC. SOC. EXP. BIOL. AND MED.,

[22] Torda, C., and Wolff, H. G., in press.

fore it has been demonstrated that the adrenotrophic hormone of the pituitary gland augments the ability of tissue to synthesize acetylcholine.

14731 P

Fractions Derived from Soy Beans and Navy Beans Which Retard Tryptic Digestion of Casein.

DONALD E. BOWMAN. (Introduced by K. K. Chen.)

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During recent studies observations were made regarding certain factors of navy and soy beans which influence digestion. While following the digestive availability of the starch of navy beans as influenced by various methods of treatment it was observed that a certain fraction of navy and soy beans retards the *in vitro* digestion of milk casein by trypsin. In view of the rather low biological value of the proteins of raw soy[1,2] and navy beans[3] and the increasing interest in their dietary use a preliminary account of some of these findings may be of interest.

Methods. In this particular work, aqueous extracts of the beans or grain in question were prepared from 1% aqueous suspensions of the finely ground materials. After centrifuging the supernatant solutions were employed in digestion experiments or were further fractionated as described.

Ten cc of such extracts or fractions prepared from this volume were added to 10 cc of a 1.52% solution of casein. Following incubation with 2 cc of 0.5% pancreatin and 2 cc of 0.8 M phosphate buffer (pH 7) for 1.5 hours the undigested protein was precipitated with trichloracetic acid and was estimated by nitrogen determination.

Results. The data of Table I represent typical findings. It will be observed that the original aqueous extracts prepared from navy and soy beans considerably retard the digestion of casein under the conditions employed while comparable extracts prepared from wheat and corn have little influence. Each value in the table represents the average of 6 determinations. The retarded digestion caused by the extracts of both types of beans becomes even more apparent when the undigested fraction is expressed in each case in terms of the percent of the total casein which is not digested as the specific result of interference by the extract. In the present series these values are 84, 51, 0.7, and 2.5 respectively for the extracts of navy beans, soy beans, wheat, and corn.

After discarding the proteins which precipitate from the original aqueous extracts of both soy and navy beans at pH 4, the filtrates obtained were found to contain nearly all of the retarding fraction. The protein content of 10 cc of such filtrates is equivalent to approximately 1 mg. Comparing this value with those in Table I it would appear that the retarding influence of the original aqueous extracts of the beans is not a function of their total protein content.

The digestion-retarding fraction can be very largely precipitated from the pH 4 filtrate of the navy bean extract with 90% acetone or ethyl alcohol. A similar precipitation of the soy bean factor may be obtained with acetone but a large part is lost with alcohol. Fractional precipitation further concentrates the material in question to a considerable degree, a small amount serving to retard the digestion of a relatively large amount of casein.

Boiling the supernatant solution of the

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, **32**, 369.

[2] Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

[3] Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, 1920, **41**, 379.

TABLE I.
Influence of Aqueous Extracts of Beans, Wheat, and Corn on the Digestion of Casein.

Extract employed	Casein employed, mg	Protein added in extract, mg	Total protein of substrate, mg	Undigested protein if extract digested alone, mg	Total undigested protein, mg	% of total protein undigested
Casein control	152	0	152	—	9.36	6.1
Navy Bean	152	20.3	173	13.1	151	87
Soy Bean	152	34.7	187	20	107	57
Wheat	152	5.6	158	2.1	12.7	8
Corn	152	1.3	154	1.3	14.4	9.4

original aqueous extract of navy beans at pH 4 and that of soy beans at pH 4 to 5 for 15 minutes decreases the activities of the inhibiting fraction to about one-fourth of the original values. Smaller degrees of inactivation were observed from pH 1 to 7 with a secondary point of increased inactivation at pH 1.

Preliminary observations indicate that bacterial proteolysis is also retarded by this substance.

In view of the behavior of this material and its heat labile properties it is of particular interest to again note the observations of Osborne and Mendel,[1] Hayward, Steenbock, and Bohstedt[2] and others who have called attention to the low nutritive value of the proteins of raw soy beans as compared with other proteins and the marked improvement in their biological value with cooking or heating. Johns and Finks[3] have made similar observations with the proteins of navy beans. The relations between these findings and those of the present report warrants additional attention. Further studies on the characteristics of this factor are in progress.

Summary. Aqueous extracts of soy beans or navy beans contain a fraction which inhibits the *in vitro* digestion of milk casein by trypsin. The fraction can be precipitated with acetone. It is partially heat labile at pH 4. Its presence may account for the low nutritive value of raw soy and navy beans.

14732

Specific Agglutination of Murine Erythrocytes by a Pneumonitis Virus in Mice.

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Horsfall and Hahn[1,2] reported the isolation of a pneumonia virus from the lungs of normal Swiss mice. This virus is readily identified by means of neutralization tests with specific anti-sera. It has been isolated from several colonies of Swiss mice by the above authors and by later investigators.

We have recently isolated a virus similar to, if not identical with the mouse pneumonia virus of Horsfall and Hahn. The virus in question was encountered during the course of successive mouse-passages of material derived from a human pneumonic lung. It cannot be determined at the present time whether the virus isolated was present in the original human lung or whether it was derived from normal mouse lung by subsequent serial intranasal passage of mouse lung suspensions. The virus has not yet appeared in the course of 17 passages of control mouse-lung suspension obtained from our stock of Swiss mice.

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[1] Horsfall, F. L., and Hahn, R. G., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 684.

[2] Horsfall, F. L., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

The discovery by Hirst[3,4] and by McClelland and Hare[5] of the capacity of influenza virus to agglutinate red cells of chickens and certain other species introduced a new and important technic in the study of influenza virus. This agglutination phenomenon is specifically inhibited by anti-influenza virus serums. Since this original discovery certain other viruses have been found to exhibit similar activity.[6,7]

We have found that the lungs of mice infected with the virus described above contain an agent capable of agglutinating murine erythrocytes and that this agglutination is inhibited by specific anti-sera. In January, 1944, consolidated lung material was obtained at autopsy from a patient, E.S., in the Presbyterian Hospital. The clinical diagnosis in this case was Addison's disease, complicated by either influenzal pneumonia or primary atypical pneumonia. The pathological diagnosis of the lung condition was primary atypical pneumonia. Consolidated portions of the lungs were received directly from the autopsy table. A considerable amount of bloody material exuded from the cut surfaces of the lung and it was noted that there was a very striking spontaneous agglutination of the red blood cells in this exudate. A suspension was prepared from the lung, and this suspension agglutinated guinea pig red blood cells. It did not agglutinate fowl red cells. The agglutination was not inhibited by anti-influenza A (PR8) or anti-influenza B ferret sera.

A 20% suspension of the human lung was inoculated into the amniotic cavity of 11-day-old chick embryos, and after 3 days' incubation the amniotic fluid was harvested and inoculated intranasally into young Swiss mice. Areas of consolidation were observed in the lungs of these mice when they were killed, 7 days after inoculation. Serial passage was carried out at 7-day intervals. By the 5th mouse-passage a fatal pneumonia was established in mice. This pneumonia is apparently caused by a virus since the lungs of infected mice are usually free from culturable bacteria. Furthermore, mouse lung suspensions have been passed through a Berkefeld V-filter and the bacteria-free filtrate when inoculated intranasally in mice has reproduced the observed pneumonitis. Dr. Horsfall has kindly supplied us with anti-PVM (pneumonia virus of mice) rabbit serum, and it has been found that this serum neutralizes the E.S. virus completely at a serum dilution of 1:125 and partially at a dilution of 1:625. The degree of neutralization compared closely in titer with that of a rabbit anti-serum prepared from the homologous E.S. strain of virus and identified the E.S. virus with PVM. When the E.S. virus from mouse lungs was inoculated into the amniotic cavity of developing chick-embryos, it survived for at least 2 days since the amniotic fluid at this time was infective for mice. On the 11th mouse passage the observation was made that the consolidated lungs of mice dying 4-8 days after inoculation produced marked agglutination of the mouse red blood cells exuding from the cut portion of the lung. This agglutination was very striking in Petri dishes in which portions of lungs were placed, but extracts prepared from suspensions of lung made in the usual manner gave only weak agglutination, or none at all. By freezing the lungs, immediately upon removal, and later heating to 80°C an extract can be prepared which gives good agglutination of murine erythrocytes. Agglutinating extracts used in the tests have been prepared in the following manner:

Portions of lungs are removed and frozen immediately. The lungs are kept at –76°C overnight, then removed, placed in a test tube and heated to 75°C for 10 minutes. They are then ground to a smooth paste and 1 cc of saline for each pair of lungs is added. The suspension is then heated at 80°C for 5 minutes, centrifuged, and the supernatant fluid

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[3] Hirst, G. K., *Science*, 1941, **94**, 22.

[4] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.

[5] McClelland, L., and Hare, R., *Canadian Pub. Health J.*, 1941, **32**, 530.

[6] Burnet, F. M., *Aust. J. Exp. Biol. and Med. Sc.*, 1942, **20**, 81.

[7] Nagler, F. P. O., *M. J. Australia*, 1942, **1**, 281.

TABLE I.
Inhibition of Mouse Red Cell Agglutination by Specific Rabbit Anti Serum.

Serum	Serum dilutions (final)							Agglutination by the serum (control)
	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/20
Normal Rabbit No. 4/44	++	++	++	++				0
E. S. Rabbit No. 4/44	0	0	0	0	0	+	++	0
Normal R. No. 101	++	++	++	++				0
PVM R. No. 101	0	0	0	0	+	++	++	0
Normal R. No. 102	++	++	++	++				0
PVM R. No. 102	0	0	0	+	++	++	++	0
Normal R. No. 103	++	++	++	++				0
PVM R. No. 103	0	0	0	±	++	++	++	0
Normal R. No. 5/44	++	++	++	++				0
Normal Mouse Lung R. No. 5/44	++	++	++	++	++	++	++	0
Normal R. No. 6/44	++	++	++	++				0
PR8 R. No. 6/44	++	++	++	++	++	++	++	0
Normal R No. 7/44	++	++	++	++				0
Flu. B. R. No. 7/44	++	++	++	++	++	++	++	0

Quantities per tube: 0.25 ml serum dilution.
0.25 ml E.S. lung extract, diluted 1/4.
2 drops 10% suspension of mouse red cells.
Tests read at 90 minutes.
++: marked agglutination.
0: no agglutination.

removed and titrated for its capacity to agglutinate mouse red blood cells. Freshly prepared, washed, suspensions of mouse red blood cells are readily agglutinated by the supernatant fluid. There is no agglutination of rat, cotton-rat, guinea pig, fowl, or human Group O erythrocytes. The hemagglutinating agent is relatively stable. It withstands heating at 80°C for 30 minutes, but is destroyed by heating at 100°C for 5 minutes. Infectivity of the virus for mice is completely destroyed by heating at 56°C for 30 minutes. Agglutination takes place readily at room temperature. Extracts prepared in the above manner usually give good agglutination in dilutions up to 1:32 and show traces of agglutination at 1:512.

Rabbit anti-serum was prepared by the intraperitoneal inoculation of 2 cc of mouse-lung suspensions of the virus. This anti-serum inhibits the agglutination of mouse red cells to a titer of 1:640 whereas control normal serum from the same rabbit fails to prevent agglutination in a dilution of 1:20. Anti-PVM rabbit serum supplied by Dr. Horsfall also inhibits agglutination. Sera to be tested for inhibitory power are inactivated at 56°C and are absorbed with normal murine erythrocytes before use. Inhibition of agglutination closely parallels the virus neutralization titer of such sera as have been tested. An example of serum-inhibition of agglutination is shown in Table I.

To date the lungs of 72 mice dying 4-8 days after inoculation of the E.S. virus have been studied and of these only 3 have failed to show marked agglutination of mouse red cells. In mice killed 7 days after inoculation the agglutinating phenomenon is less regular. However, lungs showing extensive consolidation produce agglutination whereas lungs with small lesions usually fail to do so. Dr. Horsfall kindly supplied us with strain No. 15 of his PVM virus. In the course of a few passages of this strain a similar agglutination of murine erythrocytes in the lungs of approximately 30% of mice dying from the infection or killed 7 days after inoculation has been observed. The agglutination phenomenon with this strain has been irregular and as yet extracts prepared from the lungs of mice inoculated with PVM virus have only low hemagglutinin titer.

Freshly removed lungs from 30 normal Swiss mice, 20 mice infected with Influenza A and 10 mice infected with Influenza B have failed to cause agglutination of mouse red cells. Lung extracts prepared from normal Swiss mice have also given negative results.

Summary. A pneumonitis virus has been isolated during the serial passage of human lung material through Swiss mice. This virus is closely related to the mouse pneumonia virus of Horsfall. The lungs of mice infected with this virus contain an agent capable of agglutinating murine erythrocytes. This agglutination is inhibited by specific anti-sera.

14733

Observations on Alloxan Diabetes.

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Since the production of necrosis of the islands of Langerhans by alloxan was first reported by Dunn, Sheehan, and McLetchie,[1] permanent diabetes has been produced in rabbits by Bailey and Bailey,[2] in rats by Dunn and McLetchie,[3] and in dogs by Goldner and Gomori.[4] The literature on this subject has been summarized by Joslin[5] and by Bailey, Bailey, and Leech.[6] From our observations confirming the production of alloxan diabetes, we have tried to select those which supplement the data in the literature.

Methods. Normal male rabbits were used and were permitted to eat up to the time of injection. A 2 or 5% aqueous solution of alloxan was injected into the ear vein either in a single dose or in 2 or 3 divided doses 15 to 30 minutes apart. Other routes of administration were tried unsuccessfully. During the next 12 hours some of the animals were given varying quantities of 50% glucose by vein as recommended by Bailey and Bailey.[2] Others were furnished an ample supply of food. In most cases these rabbits ate enough to combat the hypoglycemic stage of alloxan poisoning first observed by Jacobs.[7] Hyperglycemia and glycosuria were noted on the following day and generally reached their greatest severity by the 3rd or 4th day after injection. Once established, the diabetes has persisted throughout the period of observation (maximum 9 months).

All animals were kept in metabolism cages and daily urine collections were made, thymol being used as a preservative. The diet consisted of weighed amounts of cabbage, carrots, and oats. The available carbohydrate of the diet was calculated from standard tables, but no attempt was made to estimate the calories available from cellulose. According to Lusk[8] 22% of cellulose may be digested by the rabbit. However, the figures given for fiber content of cabbage and carrots is 1% so that there would be a relatively small correction, approximately 2% of the usual value for available carbohydrate.

Others have observed that effective doses of alloxan were generally fatal to animals under barbiturate anesthesia. The few dogs,

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[1] Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet,* 1943, **1**, 484.

[2] Bailey, C. C., and Bailey, O. T., *J. A. M. A.,* 1943, **122**, 1165.

[3] Dunn, J. S., and McLetchie, N. G. B., *Lancet,* 1943, **2**, 384.

[4] Goldner, M. G., and Gomori, G., *Endocrinology,* 1943, **33**, 297.

[5] Joslin, E. P., *New Eng. J. Med.,* 1944, **230**, 425.

[6] Bailey, C. C., Bailey, O. T., and Leech, R. S., *New Eng. J. Med.,* 1944, **230**, 533.

[7] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 407.

[8] Lusk, G., *Science of Nutrition,* W. B. Saunders Co., Philadelphia, 1931.

TABLE I.
Incidence and Duration of Alloxan Diabetes in Rabbits.

No. of rabbits	Rabbit No.	Dose of alloxan, mg per kg	Duration of diabetes, days
15		200	0-4
12		200	7-280
	28	25-175 (7 days)	107
	32	50, 0, 100	48
	33	25, 50, 50, 50	101
	35	25-75 (16 days)	72
	36	25 (10 days), 0 (4 days), 100	73
	39	50, 50, 50, 50	34
	40	25, 25, 25, 25	not diabetic
	41	25, 25, 25, 25	” ”

cats, and rabbits injected with alloxan intravenously under pentobarbital anesthesia in this laboratory all died. However, it was possible to give full doses (200 mg per kg) to rabbits under light ether anesthesia with no increase in the mortality rate.

Results. Diabetes has been produced by various doses of alloxan (Table I). When 200 mg per kg was given only 12 of 27 rabbits survived more than 4 days. The repeated daily injection of smaller doses was less lethal but 2 deaths in this series are not tabulated. Hard and Carr[9] using single doses of 50 mg per kg of alloxan made one of 5 rabbits diabetic. When they used 100 mg per kg, 8 of 13 rabbits became diabetic, and our results are in good agreement with theirs.

It is clear that the effective dose per day can be lowered somewhat by giving the drug for several days. Rabbits 33 and 39 given 50 mg per kg per day resemble the 3 rabbits made diabetic with 40 mg per kg per day by Bailey *et al.*[6] Hard and Carr[9] state that a refractory animal usually does not respond to successive doses of alloxan, a phenomenon observed in this laboratory also. Goldner and Gomori[4] note that dogs treated with an ineffective first dose show an increased resistance to alloxan. Whatever causes an occasional animal to be refractory, this failure to respond has not been induced in rabbits by repeated small or ascending doses. There are recognized differences in the susceptibility of different animals and of different species[10] to alloxan, but in a single animal it appears that a minimal effective concentration of the drug can cause progressive damage which after several days leads to manifest diabetes. One cannot exclude the possibility that alloxan is destroyed or excreted slowly so that it has a cumulative action, but this seems unlikely when the results of its injection by other routes are considered. It is suggested that it is not the accumulation of the drug but the extension of the pancreatic injury which accounts for this effect.

Alloxan in doses up to 200 mg per kg subcutaneously and in amounts of 300 and 700 (2 rabbits) mg per kg intraperitoneally has caused no evidence of diabetes, renal injury or anemia. It seems that in the rabbit, alloxan is ineffective except by the intravenous route, a finding which suggests that it is rapidly altered when in contact with the tissues. However, in the rat diabetes has been produced by alloxan administered subcutaneously or intraperitoneally[3,11] and the stability of this compound in the body has not yet been studied.

Observations on the Mechanism of Alloxan Action. Table II shows that insulin mixed with 2% alloxan *in vitro* is not inactivated. These results emphasize those of Goldner and Gomori[12] because the smaller dose of insulin (0.2 U per kg) used was still effective.

In order to show that alloxan damages the cells of the islands of Langerhans and that the subsequent liberation of insulin produces the

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[9] Hard, W. L., and Carr, C. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 214.

[10] Brunschwig, A., and Allen, J. G., *Cancer Research*, 1944, **4**, 45.

[11] Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 287.

[12] Goldner, M. G., and Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 73.

TABLE II.
Response to Insulin (0.2 units per kg intravenously) in 2% Alloxan.

	Blood sugar						
		Min					
	Before mg/100 cc	15 mg/100 cc	30 mg/100 cc	45 mg/100 cc	60 mg/100 cc	Fall* %	Alloxan, mg/kg
	Insulin in Alloxan—6 tests.						
Avg	112	101	74	86	84	38	7
Range	100-129	84-114	61-84	69-107	72-117	29-53	
	Insulin alone—48 tests.[17]						
Avg	100	82	60	65	68	44	0

* The difference between the initial blood sugar and the lowest blood sugar observed during each test has been expressed as the percent fall from the initial level.

TABLE III.
Response of Blood Sugar to Alloxan in Normal and Diabetic Rabbits.

			Blood sugar					
				Hr after injection				
Rabbit No.	Condition	Dose of Alloxan (intravenous) mg/kg	Before inj. mg/100 cc	2 mg/100 cc	4 mg/100 cc	6 mg/100 cc	8 mg/100 cc	Ref.
42	Normal	200	114	201	174	56	72	
43		200	108	196	172	42	53	
		200	95	170	80	70	32	(18)
		210	95		50	30	—	(7)
		214	112		205	90	32	(7)
		200	108	237	80	28	—	(12)
		200	102	378	57	40	—	(12)
		150	123	306	—	32		(12)
Avg			107	248	116	48	47	
19	Diabetic fasted	None	510	432	394	362	397	
19			361		353		361	
28			417				318	
32			460				492	
33			285				183	
33			372		361		268	
36			514		430		441	
Avg			417		384		351	
5	Diabetic fasted	200	343	367	364	293	252	
19		200	426			426	397	
28		200	351	362	373	341		
32		200	380	361	352	308	299	
33		200	305	271	234	202	197	
33		200	432			397	374	
36		200	362			435	397	
Avg			371	340	330	343	319	

hypoglycemic phase, alloxan has been given to rabbits with well established alloxan diabetes.* Table III presents these experiments. The control studies included the demonstration of the hypoglycemic effect of 200 mg per kg of alloxan in normal fasted animals. Normal rabbits 42 and 43 agreed with the results of this dosage found in the literature, which have been included for completeness. The figures cited from Jacobs[7] were estimated from his curves. When diabetic rabbits were fasted

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[17] Dohan, F. C., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 24.

* Ridout, Ham, and Wrenshall have just reported 2 such experiments in dogs which yielded similar results. (*Science*, 1944, **100**, 57.)

TABLE IV.
Average Fasting Urinary Excretion of Glucose and Nitrogen.

No. rabbits	Procedure	Urine glucose, g/kg/day	Urine nitrogen	
			Avg, g/kg /day	Range, g/kg/day
5*	Normal	0	0.56 ± 0.11	0.35-1.0
	” 13	0	0.60†	
	” 14	0	0.48†	
7	Alloxan Diab.	1.1	0.78 ± 0.20	0.32-1.80
12‡	Phlorhizin[16]	2.3 ± 0.13	0.77 ± 0.05	0.30-1.20

* The average daily excretion of 4-day fasting periods and S. D. of mean.
† Calculated from statement in text; data not reported.
‡ The averages were calculated from 24 daily determinations on 12 rabbits fasted 1-4 days.

TABLE V.
Effect of Fasting on Blood Sugar of Rabbits.

No. rabbits	Blood sugar			
	Beginning of fast		End of fast	
	Avg mg/100 cc	Range mg/100 cc	Avg mg/100 cc	Range mg/100 cc
5 Normal	113	99-126	109	98-124
7 Diabetic	367	314-536	137	85-202

the blood sugar fell appreciably in the course of 8 hours as shown in the second group of experiments. In diabetic animals given alloxan there was no greater fall than when such rabbits were simply fasted. This adds another type of experiment which supports the concept that the hypoglycemia of alloxan is due to the liberation of pancreatic insulin. In addition, the daily glycosuria of these animals did not increase after the second dose of alloxan.

Metabolic Studies. Little is known about diabetes in the rabbit and the fasting metabolism of this species has not been reported. Table IV presents our findings on fasted diabetic rabbits and includes the data encountered in the literature. The nitrogen excretion of fasted normal rabbits in this study agrees with the statements in the text made by Greeley and Drury[13] and Drury.[14] Greeley[15] described total pancreatectomy in the rabbit but reported no metabolic studies by which it could be compared with other types of diabetes or with other species. In alloxan diabetes, the low excretion of glucose was due to the fact that all but one animal became free of glycosuria before the fourth day of fasting. This improvement was also measured by the fall in blood sugar (Table V) during 4-day fasting periods. The average nitrogen excretion of diabetic animals was not strikingly increased above the normal level. Lusk's[16] data on phlorhizin glycosuria in rabbits provide an interesting comparison. The glycosuria is distinctly greater, the nitrogen excretion about the same as in alloxan diabetes. The significance of these findings will be discussed later.

One observation about the excretion of nitrogen by the rabbit deserves mention. We have found that during a 4-day fast, both normal and diabetic animals, like Lusk's phlorhizinized rabbits, showed great daily irregularities of nitrogen excretion. This exceeds the fluctuations of other species such as the dog, cat, pig, and goat and was not corrected by daily emptying of the bladder by

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[13] Greeley, P. O., and Drury, D. R., *Am. J. Physiol.*, 1940, **130**, 249.

[14] Drury, D. R., *J. Clin. Endocrinology*, 1942, **2**, 421.

[15] Greeley, P. O., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 309.

[16] Lusk, G., *Z. f. Biol.*, 1898, **36**, 82.

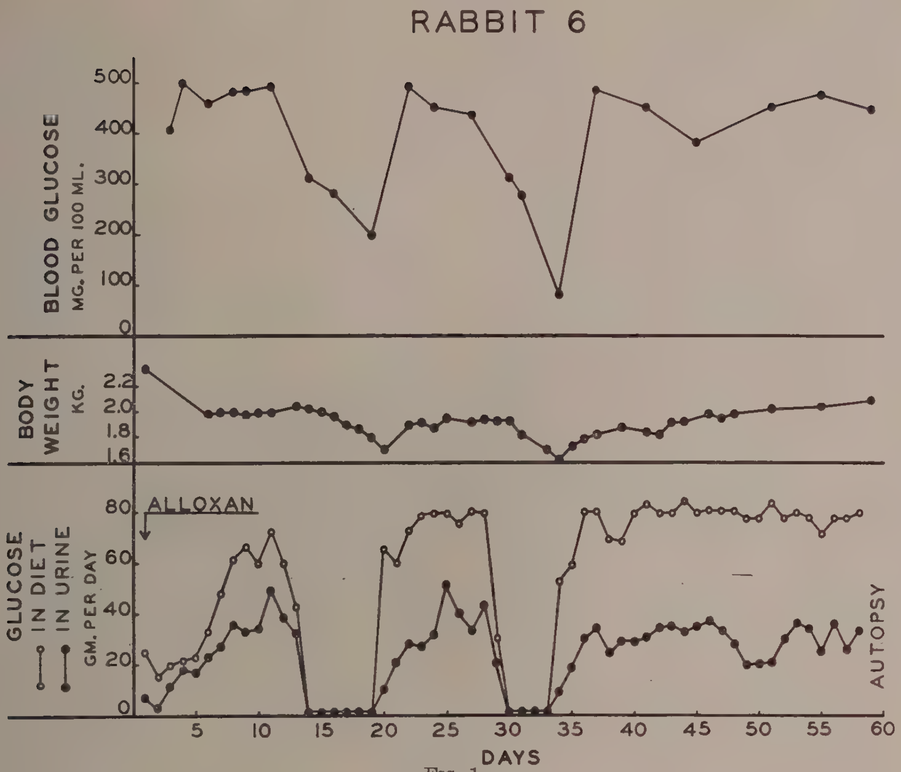

FIG. 1.

Course and severity of diabetes after 200 mg per kg of alloxan had been given on the first day charted. The slight glycosuria on this day was attributed to intravenous glucose. The periods when glycosuria ceased were due to fasting. Note capacity to gain weight in the last days of observation. No insulin was given.

pressure. This behavior of the urinary nitrogen excretion makes the rabbit a relatively poor animal for metabolic studies.

The proportion of the available dietary glucose excreted in the urine has also been used as an index of the severity of the diabetes. Our animals have excreted from 15 to 60% of the available glucose of the diet, the majority being about 40-50%. Fig. 1 illustrates the relationship between food intake and glycosuria in a typical case. The decline of glycosuria during fasting is also noted. About half of the rabbits occasionally had slight ketonuria. In 4 rabbits the diabetes was controlled by insulin for periods from 15 to 27 days. There was no evidence of improvement in the diabetes as a result of this control.

Other Observations. The incidence of early necrosis and subsequent atrophy of the islands and the occurrence of renal damage are summarized in Table VI and need no comment. However, hydropic degeneration has been seen in 2 rabbits examined after diabetes of 48 and 58 days duration. At this time the hydropic change was associated with atrophy and this differs from the hydropic islands described early in the disease by Bailey *et al.*[6] Three animals with prolonged diabetes had normal levels of blood urea nitrogen; 2 which died in 7 and 10 days had elevated values. During the first few days after alloxan the specific gravity of the urine was low (1015) but it returned to normal (1025 to 1055) in 6 animals tested during the chronic stage of diabetes. These changes have not been clearly related to the usually minor pathological alterations in the kidneys.

TABLE VI.
Lesions of Alloxan Diabetes in Rabbits.

Days after alloxan	No. of rabbits	Pancreas			Renal tubules		
		Necrosis	Atrophy	Hydropic	Necrosis	Atrophy	Normal
0-7	7	7	1	0	6	1	0
8-86	12	1	12	2	2	5	5

Hemoglobinemia and hemoglobinuria were observed in a few of our rabbits (*cf.* [1]). Occasional hemoglobin and erythrocyte counts showed that the red blood cells fell to 1 to 2 million a few days after a single large dose and the blood count became normal in about 3 weeks. In the few animals with blood counts, diabetes has not been produced except by a dose of alloxan sufficient to cause anemia.

No sign of cataract formation has been found in 11 rabbits which were diabetic for 6 weeks or longer. This is in contrast to the observation of Bailey *et al.*[6] that cataracts developed in 4 to 6 weeks in rabbits with alloxan diabetes.

Finally, attempts to produce diabetes with nitrates, methylene blue, and sodium tartrate have been entirely negative, the last in spite of its severe action on the renal tubules which is not unlike the milder effect of alloxan.

Discussion. In connection with the results shown in Tables II and III, the findings relevant to the mechanism of action of alloxan may be reviewed. (a) There is transitory hyperglycemia followed by hypoglycemia at 6-12 hours which in turn is followed by diabetes. (b) Alloxan does not inactivate insulin *in vitro*. (c) Although the initial hyperglycemia can be prevented by insulin or phlorhizin, this does not protect the island from necrosis.[12] (d) There is a quantitative similarity between the hypoglycemic effect of alloxan and that of a dose of insulin equivalent to the insulin content of the pancreas.[18] (e) Necrosis of the islands may be apparent in 12 hours or less. (f) There is an absence of the hypoglycemic phase and of any change in the severity of the diabetes, when alloxan is given to diabetic rabbits. All the findings indicate that alloxan injures the cells of the islands of Langerhans with the resultant liberation of insulin which then causes hypoglycemia. The irreversibly damaged cells fail to secrete insulin and diabetes ensues. Alloxan might cause hypoglycemia by temporary injury to the liver. However, its failure to do so in diabetic animals would seem to exclude such an action and to support the hypothesis that damage of the islands is largely, if not entirely, responsible for the results.

The characteristic lesion of alloxan diabetes is atrophy of the islands of Langerhans. The beta cells are particularly vulnerable and a few alpha cells usually remain.[4,19] It is noteworthy that the acute necrosis caused by alloxan and the slower destruction of the beta cells in pituitary-diabetes[20] lead to an essentially similar end result. There has been no recovery from alloxan diabetes treated by insulin,[2,6] a fact which is in agreement with all previous experience with atrophy of the islands.[20] The occurrence of hydropic degeneration in conjunction with island injury appears to be comparable to its development after partial pancreatectomy,[21] and in pituitary-diabetes. Based on previous work, we interpret this finding as indicating that enough beta cells were destroyed by alloxan to make the animals diabetic. The surviving beta cells subsequently became hydropic because of the hyperglycemia. Hydropic degeneration is not seen more frequently (a) because almost all of the beta cells are usually destroyed by alloxan or (b) because of the time when the islands have been examined for this transitory lesion. The occurrence of hydropic degeneration may account for the one dog in which recovery from alloxan diabetes was reported by Carrasco-Formiguera.[22]

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[18] Hughes, H., Ware, L. L., and Young, F. G., *Lancet*, 1944, **1**, 148.

[19] Dunn, J. S., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. and Bact.*, 1943, **55**, 245.

[20] Lukens, F. D. W., and Dohan, F. C., *Endocrinology*, 1942, **30**, 175.

[21] Allen, F. M., *J. Metab. Res.*, 1922, **1**, 5.

The metabolism of diabetic rabbits requires comment. When fed they excrete only a moderate amount of carbohydrate. On fasting the glycosuria ceases, there is no ketonuria, and the nitrogen excretion is somewhat greater than that of normal fasted rabbits. These facts are compatible with partial pancreatic diabetes, and the persistence of some islet tissue makes this a probable explanation. However, pancreatic diabetes as seen in the goat[23] prompts one to ask whether alloxan may not produce a severe diabetes similar to that following pancreatectomy. In depancreatized goats there is no glycosuria or ketonuria on fasting and little or none when they are fed an ample amount of greens. After pancreatectomy or phlorhizin the nitrogen excretion is approximately equal in the goat and it is the same after alloxan or phlorhizin in the rabbit. It is thus possible that the seemingly mild diabetes of the rabbit may at times be severe diabetes for this species.

Summary. Diabetes has been produced in rabbits by the intravenous injection of alloxan.[1,2] The effectiveness of the repeated daily injections of doses as low as 50 mg per kg indicates that alloxan has a cumulative action or that a gradual extension of pancreatic injury takes place.

When alloxan was given to rabbits with alloxan diabetes no effect on the blood sugar was observed. This supports the favored hypothesis that the hypoglycemic phase of alloxan poisoning is due to the liberation of insulin from damaged cells.

Metabolic studies indicate that alloxan diabetes in rabbits corresponds to partial pancreatic diabetes. In this connection the factor of species is discussed.

Hydropic degeneration was observed in the islet tissue of 2 animals. This change has been attributed to the effect of hyperglycemia on beta cells which escaped destruction by alloxan.

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[22] Carrasco-Formiguera, R., *J. Lab. and Clin. Med.*, 1944, **29**, 510.

[23] Lukens, F. D. W., *Am. J. Physiol.*, 1938, **122**, 729.

14734

Bacteriostatic Action of Sulfonamide-Penicillin and Urea-Penicillin Mixtures *in vitro.*

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The effectiveness of penicillin* in the treatment of certain types of infections has become well established. In some instances, however, particularly general sepsis and local wound infections, it is possible that combinations of penicillin with other chemo-therapeutic agents will prevent unsuccessful results that are occasionally encountered with penicillin alone. The situation is analogous to that of the sulfonamides, in which urea-sulfonamide mixtures are highly effective for topical application to infected wounds.

The following experiments were designed to study the bacteriostatic action of sulfonamide-penicillin and urea-penicillin mixtures *in vitro.*

Method. Growth curves were measured turbidimetrically under rigidly controlled conditions for a period of 24 hours, according to a method developed in this laboratory and described elsewhere in detail.[1] In the present studies the organisms were a coagulase-positive strain of *Staphylococcus aureus,* and a

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* The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

[1] Kirby, W. M. M., and Rantz, L. A., *J. Exp. Med.*, 1943, **77**, 29.

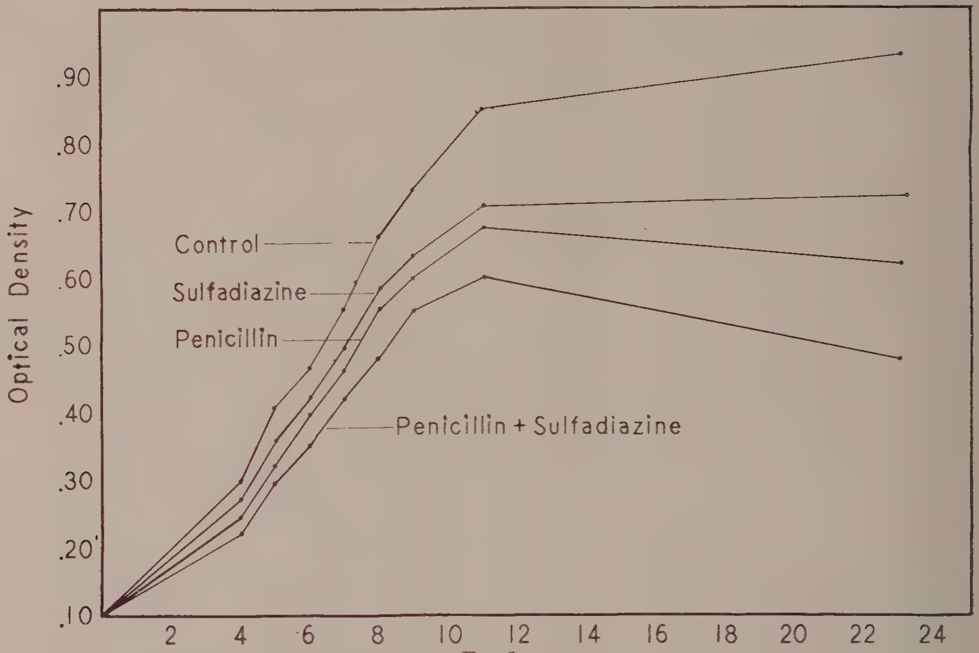

FIG. 1.
The effect of penicillin and sulfadiazine separately and in mixture upon the growth of *Staphylococcus aureus.*

Lancefield Group A hemolytic streptococcus. For the staphylococcus, 0.5% peptone was added to the synthetic medium previously described, and the streptococcus was grown in beef infusion broth. The pH of the media was not altered by the addition of urea and sulfonamide solutions.

Concentrations of penicillin, sulfadiazine, and urea that produced moderate bacteriostasis during the stage of logarithmic multiplication of the organisms were determined, and added singly and in mixtures to standardized cultures for the various experiments. For one staphylococcus urea 2%, sulfadiazine 50 mg % and penicillin 0.01 unit per cc were used, and for the streptococcus penicillin 0.05 u./cc, sulfadiazine 100 mg %, and urea 2% were found to produce adequate bacteriostasis. The number of viable bacteria at the beginning of each experiment was, for both organisms, approximately one million per cc.

Results. Sulfadiazine, urea, and penicillin in concentrations which were not bacteriostatic separately, did not cause inhibition of growth when mixed together. If concentrations were used that were bacteriostatic separately, sulfonamide-penicillin and urea-penicillin mixtures produced greater bacteriostasis than did any of these substances alone. However, in no instance was the bacteriostatic action of the mixture greater than the sum of the inhibitory effects of the individual constituents. In other words, there was no actual potentiation of penicillin action. Fig. 1 illustrates this for the staphylococcus. It is also evident from the figure that lysis of staphylococci was no greater with the mixtures than with penicillin alone.

Discussion. The results of these experiments are similar to those found previously with urea-sulfonamide mixtures; namely, that the bacteriostatic action of the mixtures represents merely the additive effects of the separate constituents.

Divergent views have been expressed concerning urea-sulfonamide mixtures,[2,3] largely as a result of differences in technical methods and in interpretation. In the opinion of the present author true potentiation can be best demonstrated when the following conditions are fulfilled: (1) The medium should support luxuriant growth of the organisms, (2) measurements of bacteriostasis should be made frequently during the phase of active growth, and (3) concentrations of the bacteriostatic agents should not be so great in relation to the size of the inoculum that, either separately or in combination, active growth of the bacteria is prevented. The last point is especially important and requires further elucidation. When the bacteriostatic action of the mixture is too strong good growth of the organism never occurs; the effect is that of rendering the medium unsatisfactory. Under these circumstances valid comparisons cannot be made with experiments in which there is good initial bacterial growth. Superficially there may appear to be true potentiation, but actually there is not. This important fact, namely, that in experiments such as these bacteria must have certain minimal conditions favoring good growth, has been previously emphasized.[4] It is believed that such pitfalls and artefacts can best be avoided by adhering to the conditions listed above. If true potentiation exists, bacteriostasis of mixtures should be greater than merely the additive effects of the separate constituents when there is active growth of all the organisms. It is clear from Fig. 1 that, under these conditions, there is no actual synergism or potentiation; the effects are merely additive.

The potential clinical significance of these observations is difficult to predict. However, in seriously ill patients who are not responding well to penicillin it would seem not unreasonable to add the effect of another bacteriostatic agent such as sulfadiazine.

Summary. ***In vitro,*** sulfonamide-penicillin and urea-penicillin mixtures have been shown to produce greater bacteriostasis than penicillin alone with the *Staphylococcus aureus* and hemolytic streptococcus. This increased inhibition represents the additive effects of the separate bacteriostatic agents; there is no actual potentiation of penicillin action.

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[2] Tsuchiya, H. M., Tenenberg, D. J., Clark, W. G., and Strakosch, E. A., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 262.

[3] Lee, S. W., Epstein, J. A., and Foley, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 105.

[4] Bloomfield, A. L., and Felty, A. R., *J. Exp. Med.*, 1924, **39**, 367.

14735

Folic Acid and Biotin Synthesis by Sulfonamide-sensitive and Sulfonamide-resistant Strains of *Escherichia coli.*

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When certain poorly absorbed sulfonamides, such as succinylsulfathiazole or sulfaguanidine, are incorporated in highly purified diets and fed to rats, bacteriological studies show the total number of organisms in the feces of such rats to be unaffected by the presence of the sulfonamide, although certain types of bacteria, such as the coliform organisms, may diminish or even disappear.[1] The feeding of such sulfonamide rations to weanling rats also results in the production of a nutritional deficiency[2,3] which may be corrected by feeding the animals biotin and folic acid.[4]

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[1] Gant, O. K., Ransone, B., McCoy, E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 276.

[2] Welch, A. D., *Federation Proc.*, 1942, **1**, 171.

[3] Black, S., Overman, R. S., Elvehjem, C. A., and Link, K., *J. Biol. Chem.*, 1942, **145**, 137.

Black, McKibbin, and Elvehjem[5] originally postulated that such sulfonamides owe their ability to induce nutritional deficiencies to a depletion in the intestine of organisms capable of synthesizing B vitamins. More recently, however, Gant, Ransone, McCoy and Elvehjem[1] suggested that strains of *Escherichia coli* in the rat intestine may become resistant to the action of succinylsulfathiazole and that such resistant strains have probably lost much of their ability to synthesize growth factors such as biotin and folic acid.

We have studied the *in vitro* production of biotin and folic acid by *E. coli* to determine whether the state of sulfonamide resistance of the organism affected its ability to synthesize these vitamins.

Procedure. Several attempts to isolate a sulfonamide-resistant form of *E. coli* from the feces of rats which had been fed a purified diet containing 2% succinylsulfathiazole for at least three weeks proved unsuccessful. Therefore a laboratory strain of *E. coli* was treated in the following manner. From our original culture of the organism two strains were carried. One strain, designated "Normal," was transferred daily in a medium of the following composition: bacto-peptone, 1.0%; NaCl, 0.6%; glucose, 0.2%; K_2HPO_4, 0.1%. The other strain was carried in the bacto-peptone medium containing amounts of sulfanilamide that were gradually increased until the daily transfers could be made in medium containing M/100 drug. This strain was then called "Resistant."

The procedure for determining the amounts of biotin and folic acid synthesized by the "Normal" and "Resistant" strains was as follows: Twenty ml quantities of the bacto-peptone medium containing varying amounts of sulfanilamide were placed in 125 ml Erlenmeyer flasks and were sterilized by autoclaving at 12 lb pressure for 5 minutes. One ml of a 24-hour culture of the "Normal" strain in bacto-peptone medium or one ml of a 24-hour culture of the "Resistant" strain in bacto-peptone medium containing M/100 sulfanilamide was used as inoculum for each flask. The flasks were incubated 20 hours at 37°C, turbidities were determined, and the cultures were autoclaved to stop growth.

In the determination of folic acid the cultures were assayed both before and after a 24-hour period of takadiastase digestion. The assay organism was *Streptococcus fecalis* (*Streptococcus lactis R*), using the method described by Mitchell and Snell.[6] The folic acid standard was a solution of crystalline *Lactobacillus casei* factor kindly provided by the Lederle Laboratories.

In preparation for the biotin assays one ml of culture was autoclaved in the presence of one ml of 6N H_2SO_4 for one hour at 15 lb pressure. The assay organism was *L. casei e*, used according to the method of Landy and Dicken.[7] A solution of crystalline biotin served as a standard.

Turbidities of the cultures were read in a Klett-Summerson photoelectric colorimeter with a 540 mμ filter. Assay media contained 5 mg of *p*-aminobenzoic acid in every 100 ml. Adequate controls were run to correct for the amounts of folic acid and biotin present in the uninoculated growth medium. It was determined repeatedly that the amount of sulfanilamide in the samples had no inhibitory effect on the assay organisms.

Results. The results of 2 experiments are given in Table I. These assays indicate that the amount of folic acid produced in a culture was determined by the presence of sulfonamide in the growth medium, and not by the state of sulfonamide-resistance of the organism. That the amount of folic acid found in a given culture was not a function of the number of bacteria present was most strikingly demonstrated by the data given in Table I for the "Resistant" strain. It will be noted that this strain showed higher bacterial densities in the presence of the drug than in its absence. Folic acid synthesis, however, decreased as the concentration of sulfanilamide

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[4] Welch, A. D., and Wright, L. D., *J. Nutrition*, 1943, **25**, 555.

[5] Black, S., McKibbin, J. M., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 308.

[6] Mitchell, H. K., and Snell, E. E., *The University of Texas Publication*, 1941, **4137**, 36.

[7] Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

TABLE I.
Effect of Added Sulfonamide on Production of Folic Acid and Biotin by "Normal" and Sulfonamide "Resistant" Strains Developed from a Single Culture of *E. coli.*

Exp. No.	Strain	Molarity of sulfanilamide in growth medium	Turbidity after 20 hrs growth	Folic acid, γ/ml		Biotin γ/ml
				Untreated culture	Takadiastase treated culture	
I.	"Normal"	0	390	.018	.013	.0051
		M/100	223	.0002	.0000	.0015
		M/200	286	.0007	.0001	.0028
		M/400	315	.0012	.0007	.0030
	"Resistant"	0	330	.024	.020	.0032
		M/100	340	.0019	.0008	.0031
		M/200	350	.0083	.0059	.0040
		M/400	345	.018	.0080	.0044
II.	"Normal"	0	377	.015	.015	.0054
		M/100	210	.0000	.0004	.0011
		M/200	272	.0005	.0005	.0023
		M/400	286	.0012	.0008	.0032
	"Resistant"	0	280	.023	.023	.0037
		M/100	330	.0017	.0012	.0034
		M/200	345	.0075	.0071	.0041
		M/400	330	.015	.010	.0039

increased. Takadiastase digestion did not release extra folic acid from the culture but often decreased the amount of this vitamin that could be demonstrated by *S. fecalis* assay. This was particularly shown in the data of Experiment I.

Under the conditions of these experiments the amounts of biotin found in the cultures were not greatly influenced by the presence of sulfanilamide in the growth medium, but often varied with, and might possibly be a function of, the number of organisms present (turbidity).

Discussion. If these *in vitro* results are an indication of the *in vivo* activity of organisms in the intestinal tract of animals receiving a purified diet containing a poorly absorbed sulfonamide, it would seem that the presence of the sulfonamide in the intestine, and not the state of sulfonamide-resistance nor the total number of vitamin-synthesizing organisms present, influences the bacterial synthesis of growth factors and, thereby, the nutritional state of such animals.

Summary. Less folic acid was synthesized *in vitro* by both a sulfonamide-sensitive and by a sulfonamide-resistant strain of *Escherichia coli* when these organisms were grown in the presence of sulfanilamide than when they were grown in the same medium not containing the drug. The amount of biotin synthesized was not greatly affected by the presence of the drug in the growth medium.

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Choline Esterase Activity of Skeletal Muscle in Various Conditions.

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Very little information is available as to possible changes in acetylcholine (Ach) metabolism under pathologic conditions. The concentration of Ach as determined in tissue extracts is not a measure of its activity. Determination of the activity of choline esterase (ChE) in tissue however may be expected to yield information, though indirect, with regard to changes in the activity of the hypothetical transmitter substance.

The present study, which we intend to continue, deals with the activity of ChE in striated muscle in a number of pathologic conditions.

So far ChE activity in muscle has generally been determined per hour and per 100 mg of tissue (QChE). It has been shown by Marnay and Nachmansohn[1] that at the neuromuscular junctions of striated muscle, there is a concentration of ChE 30,000-50,000 times as high as in the surrounding muscle tissue. The distribution of the motor endplates throughout the muscle is not uniform. It is therefore obvious that a comparison based on QChE values is not valid unless the entire muscle is extracted for the determination and the weight of the whole muscle is taken into consideration. Assuming that for any given species, the same muscle, within certain variations, will contain a fixed number of motor endplates, the total choline esterase (TChE = QChE × muscle weight) will represent a comparable measure of ChE activity. If muscles of different weight are compared it must also be considered that their ChE content as shown below is not directly proportional to muscle weight.

The effect of nerve degeneration on choline esterase activity was first studied by Martini and Torda.[2] In the denervated gastrocnemius muscle of rats, they found a progressive decrease of the QChE varying from 12 to 75% from the second to the twelfth day after denervation.

Feng and Ting[3] reported a decrease of the ChE activity of 20-30% in the nerve-containing tibial portion of the toad-sartorius between the twelfth and the thirty-fourth day after section of the sciatic nerve. No changes were found in the nerve-free pelvic portion of the sartorius.

Recently it has been stated by Couteaux and Nachmansohn[4] and by Nachmansohn and Hoff[4a] that in the denervated gastrocnemius of guinea pigs there is no significant fall in the QChE during the first 2 weeks after denervation. From the third to the fourth week on, coinciding with morphological changes in the endplates, a 20-30% decrease in the QChE was said to occur at the endplates. In view of these discrepancies in the available data the ChE activity of denervated voluntary muscle has been reinvestigated. It also appeared of interest to study the effect of nutritional muscular dystrophy on the ChE activity of the muscle.[5]

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[1] Marnay, A., and Nachmansohn, D., *C. R. Soc. de biol., Paris*, 1937, **124**, 942.

[2] Martini, E., and Torda, K., *K. Wschr.*, 1937, **23**, 824.

[3] Feng, T. P., and Ting, Y. C., *Chin. J. Phys.*, 1938, **13**, 141.

[4] Couteaux, R., and Nachmansohn, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 177.

[4a] Nachmansohn, D., and Hoff, E. C., *J. Neurophys.*, 1944, **7**, 27.

[5] Nerve endings in guinea pigs with nutritional muscular dystrophy have been described as normal by Rogers, Pappenheimer, and Goettsch (*J. Exp. Med.*, 1931, **54**, 167) and in rats by Pappenheimer (*Am. J. Path.*, 1939, **15**, 179). Telford (*Anat. Rec.*, 1941, **81**, 171), however, found them reduced in number in the degenerated muscles. Reexamination with a modified Ranvier gold chloride method (Pappenheimer and Stoerk, unpublished observations) has shown complete disappearance of the hypolemmal nerve endings together with the entire motor end plate in the dystrophic fibers, although epilemmal neurites are well-preserved.

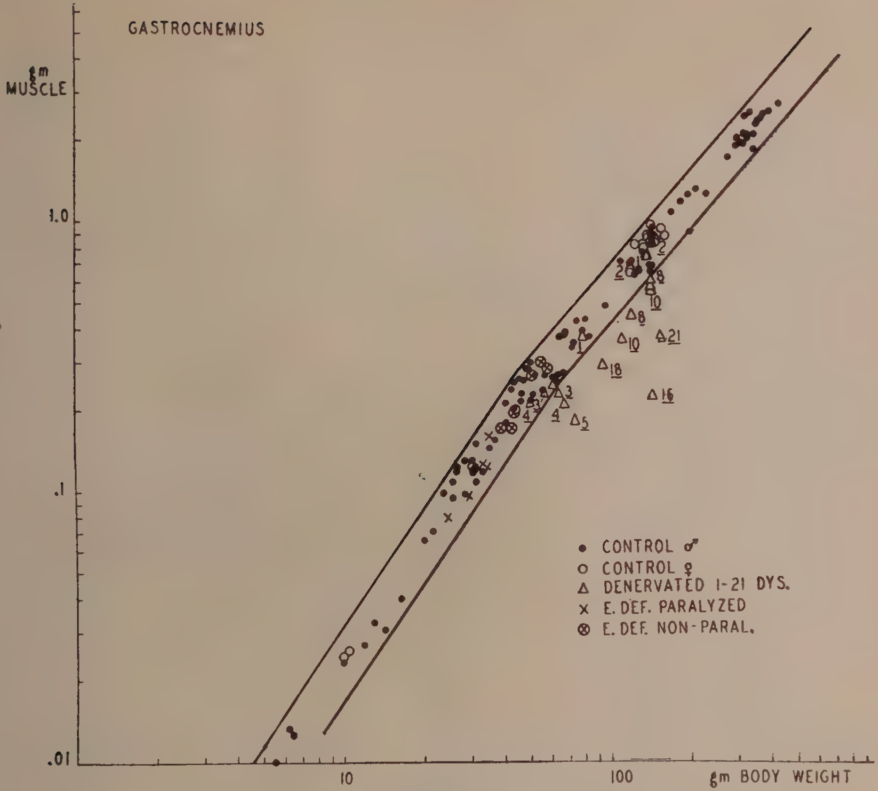

CHART 1.
Log. log. plotting of gastrocnemius weight against body weight.

Seventy apparently healthy albino rats of the Sherman strain maintained on Rockland rat pellets were used as normal controls. They varied in age from one day to 6 months and in body weight from 5.5 to 469 g. In 18 male rats the right sciatic nerve was cut and a portion of the nerve approximately 1 cm in length was removed. Muscular dystrophy was induced by a diet deficient in vitamin E in 7 young nursing rats according to the technic described by Goettsch and Ritzmann.[6] The animals were killed 2 days after the onset of paralysis. Six animals raised under identical conditions but showing no clinical or histological evidence of muscular dystrophy served as controls. All animals were killed with ether. The right gastrocnemic muscle was dissected, and weighed. The entire muscle was then extracted with Na-Mg buffer by grinding with silica. The ChE activity of the suspension was determined with the manometric method (Ammon[7]). The contralateral muscles of the animals in which the sciatic nerve was sectioned were used as controls. In all E-deficient animals the contralateral muscle was examined histologically.

A graphic presentation of the data is given in Charts I, II, and III. In Chart I gastrocnemius weight is plotted against body weight on a log., log. grid. The usual straight line relationship with a single "break" is observed

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Two days after section of the sciatic nerve, the hypolemmal axons in the gastrocnemius were also seen to have disappeared. The epilemmal axons and the sole plates showed no appreciable changes. No Wallerian degeneration had as yet occurred.

[6] Goettsch, M., and Ritzmann, J., *J. Nutr.*, 1939, **17**, 371.

[7] Ammon, R., *Arch. ges. Physiol.*, 1933, **233**, 486.

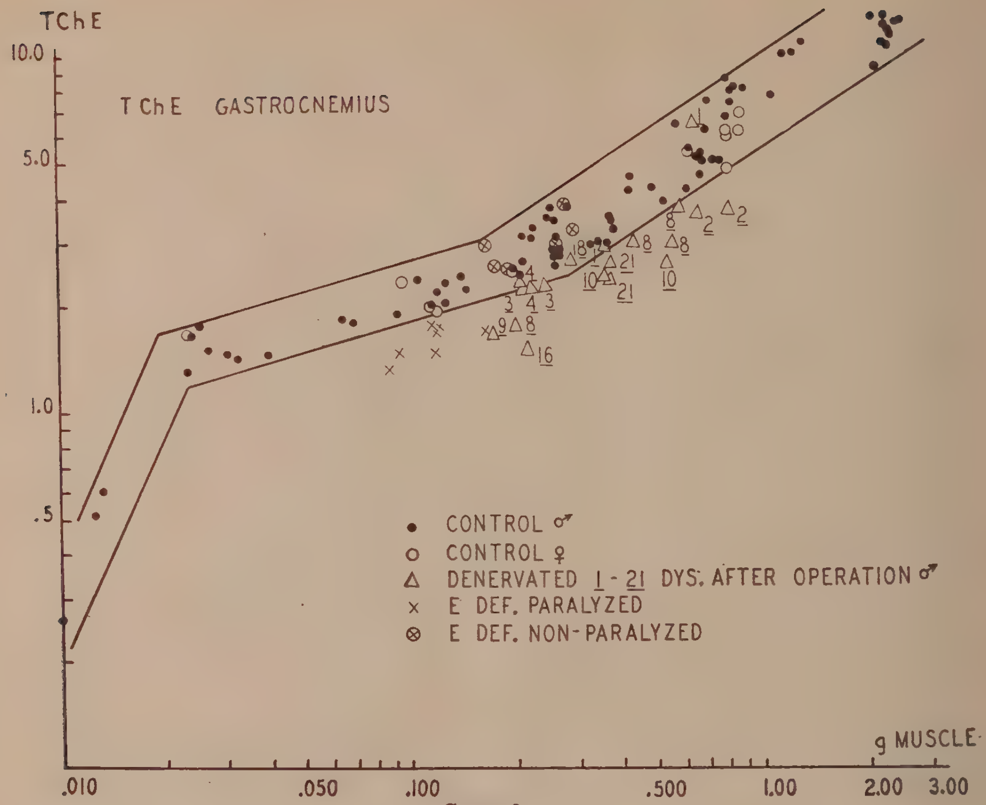

CHART 2.
Log. log. plotting TChe of gastrocnemius against gastrocnemius weight. The band includes all the controls.

as in many other organs or body constituents (Needham,[8] Zucker *et al.*[9]). The denervated gastrocnemii rather soon after section of the nerve are seen to be reduced in size. The muscular atrophy however is not progressive and it appears that in the later stages both failure of growth of the muscle and atrophic changes account for the deviation from the normal range of relative weight.

In Chart II the ChE activity of the whole muscle (TChE) is compared with the weight of the muscle on a log. scale. The width of the band includes all the data obtained from the 88 controls, expressing both biological variations and experimental error. Muscle weight and not the usual body weight has been chosen as ordinate because of the growth retardation of the muscle combined with atrophic changes occurring after denervation. the log.-log. relation obtained presents 2 "breaks" indicating changes in the rate of relative increase of the enzyme concentration. The first occurs after about 1 week of age of the animals, the second close to the onset of sexual maturity. The bulk of the TChE values in muscles denervated for more than one day, and in animals with muscular dystrophy, fall below the range of the established norm. On an average, the TChE of these two groups is reduced by approximately one-third. This reduction in the TChE of the

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[8] Needham, J., *Biochemistry and Morphogenesis*, Cambridge Univ. Press., 1942.

[9] Zucker, T. F., Zucker, L., and Sperry, W. M., *Abstracts 108th A. C. S. Meeting*, Sept., 1944, p. 2B.

TABLE I.
TChE of M. Obliqu. Inf. Oculi.

Autopsy No.	Diagnosis	Sex	Age	Muscle wt.	TChE
14,414	Meningitis	♂	18	.236	9.2
415	Lymphosa	♀	58	.138	8.2
418	Arterioscl.	♀	84	.188	14.7
419	Lymphosa	♀	73	.155	11.3
421	Ca of lung	♂	51	.325	13.9
422	Paget Dis. of bone, Pneumonia	♂	69	.201	11.3
423	Diab. mellit., Glomerulo nephr.	♀	30	.205	9.6
424	Mult. myeloma	♂	67	.211	13.3
425	Cardiac insuff.	♀	74	.177	11.9
427	Pneumonia	♂	60	.247	11.3
432	Myel. leucem.	♀	37	.151	8.8
462	Myasth. gr.	♀	30	R.235 L.231	4.9 4.6
476	Hyperthyroid	♀	42	.186	9.2

TABLE II.

No.		Cc Prostigm. (1:10,000)	Min after Ist inj.	G gastrocn. wt	TChE
Control	1	—	—	.847	5.95
”	2	—	—	.795	4.65
”	3	—	—	.855	6.75
”	4	—	—	.612	5.27
”	5	—	—	.790	6.02
”	6	—	—	.793	5.80
Exp.	7	1 × .2	30	.797	5.47
	8	1 × .4	30	.724	5.47
	9	1 × .8	died after 15	.616	4.45
	10	1 × 1.0	30	.870	5.12
	11	2 × .2	150	.905	5.78
	12	4 × .2	270	.930	4.65
	13	4 × .2	270	.850	4.25
	14	4 × .4	270	.807	3.67

All injected animals showed marked symptoms of prostigmine poisoning beginning about 10 minutes after the injection. The maximal response appeared after *ca.* 30 minutes. The effect lasted throughout the intervals in the animals which received repeated injections.

denervated muscle obviously is not a function of the time elapsed after the section of the nerve, for it is essentially the same after 2 days as after 21 days. The question as to the specificity of the fraction of the enzyme which remains active after denervation and in the dystrophic muscle may only be answered by further studies.

It seems probable that the transmission of the nerve impulses is disturbed in patients with myasthenia gravis. In 2 instances (Jones and Stadie,[10] Goodman *et al.*,[11]) the activity of ChE has been examined in muscle tissue obtained by biopsy from patients with myasthenia gravis and has been compared with that of controls. No differences were found. The comparison of small portions of muscle with regard to their ChE activity, however, as pointed out above, is open to criticism. ChE is known as a very stable enzyme. It therefore appeared safe to use tissue from autopsy material. The inferior oblique muscles of the eye were dissected from a patient who died of myasthenia gravis and in whom a large thymoma (12 x 6 x 4 cm) was present. The ChE activity of the whole muscle was determined as described above and was compared with that of muscles from patients dying from various other diseases. The findings are summarized in Table I where it is seen that the enzyme activity in the patient with myasthenia gravis was only about half as high as in the lowest of the controls. From the data on hand it cannot be decided whether or not all

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[10] Jones, M. S., and Stadie, W. C., *Quart. J. Exp. Physiol.*, 1939, **29**, 63.

[11] Goodman, L., Carlson, R. I., and Gilman, A., *J. Pharm.*, 1939, **66**, 15.

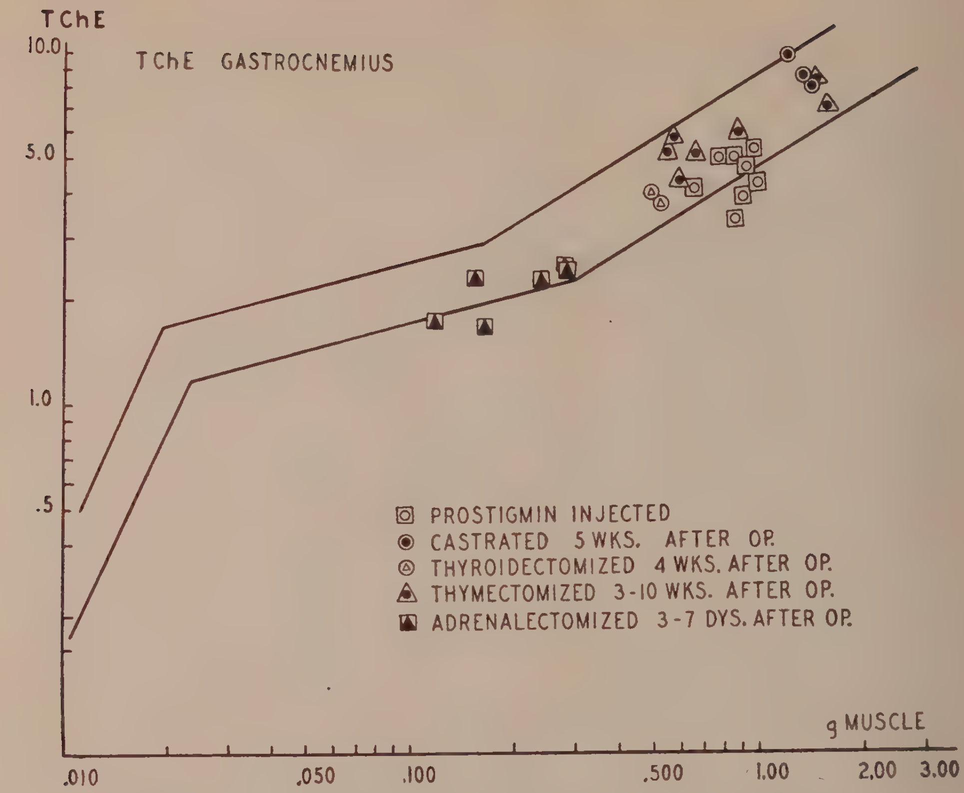

CHART 3.
Comparison of range of data (controls) from Chart 2 with experimental data.

of this reduction of the ChE activity was due to the prostigmine treatment (.5 mg almost every hour) the patient received before death. It is seen from Table II and Chart III that in rats, even the repeated injection of extremely large amounts of the drug causes only a relatively small reduction of the enzyme activity. Thymectomy in rats did not influence the activity of ChE as seen from Chart III.

In hyperthroidism an increased activity of ChE was found in the serum by Antopol *et al.*[12] The muscle ChE in a patient who died from Grave's disease (Table I) and in 2 thyroidectomized rats (Chart III) was unchanged.

Other experimental findings are summarized in Chart III and Table II. It has been shown by Glick[13] that the decrease in the ChE activity observed in the blood of prostigmine injected animals is not reflected in tissue (gastric mucosa). This is also brought out from part of our data. It was found that most marked effects of prostigmine poisoning may be observed without measurable reduction in the activity of the enzyme. Repeated large doses of prostigmine, however, did significantly lower the activity of ChE in muscle. In 3 castrated and 2 parathyroidectomized animals the enzyme activity was unchanged. That in 2 of 6 animals after adrenalectomy the TChE fell below the range of the controls appears of interest. Additional data are needed to confirm or refute the significance of this observation.

Summary. The choline esterase activity of the whole gastrocnemic muscle (TChE) in rats was found to be reduced by approximately one-third after denervation and in nutritional muscular dystrophy. It was slightly lowered after repeated injections of high doses of prostigmine and after adrenalectomy. Single injections of high doses of prostigmine had no such effect.

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[12] Antopol, W., Tuchman, L., and Schifrin, A., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 46.

[13] Glick, D., *J. Gen. Physiol.*, 1938, **21**, 297.

Thymectomy, thyroidectomy, parathyroidectomy, and castration had no effect on the TChE of the rat gastrocnemius.

The TChE of the musc. obliqu. inf. oculi in a patient who died from myasthenia gravis was found to be only half as high as that of the lowest in a series of 12 controls. It was not possible to decide whether or not all of this reduction of the enzyme activity was an effect of prostigmine treatment. The ChE activity in the muscle of a patient dying from hyperthyroidism was within the range of the controls.

We are greatly indebted to Dr. D. Nachmansohn for valuable suggestions and technical advice.

14737

Effect of Soybean Phosphatides on Utilization of the Fat-soluble Vitamins.

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We reported[1] that the utilization of vitamin A and carotene in the rat is influenced by an as yet unidentified factor present in soy bean lecithin. Patrick and Morgan[2] confirmed our findings in regard to the chick and found that the same factor influences vitamin E utilization as well as vitamin A metabolism. They also showed that yeast, extracted with certain fat solvents, was lacking in this specific factor. Earlier, Polskin[3] reported that lecithin plays an important role in the conservation of the hepatic vitamin A reserves of the chick.

Jensen, Hickman, and Harris[4] have questioned our findings on the grounds that soy bean lecithin contains a certain amount of vitamin E and they express the belief that the observed effects were due to the increased vitamin E intake provided by soybean lecithin in our diet. They could obtain no marked difference from addition of soybean lecithin to the diet of the rats in their experiments. Apparently they overlooked our suggestion that the unknown factor is contained in yeast, which was proved by Patrick and Morgan. Inasmuch as the basic diet of Jensen, Hickman, and Harris contained 8% yeast, their observations do not bear upon the problem under consideration.

To exclude the possibility that the effects of the unknown factor may be attributed to vitamin E, the following experiments were carried out in which the amount of vitamin E contained in soy bean lecithin was taken into consideration. The vitamin E content of soy bean lecithin* was determined according to the method of Emmerie and Engel, and Scudi and Buhs, as 1.3 mg per g.

Experimental. Female albino rats, Sherman strain from our laboratory colony, 28 to 30 days old, weighing approximately 52 to 60 g were kept in individual metal cages with wire mesh floors. The basal diet fed had the following percentage composition: casein, Labco, 20; cerelose, 72; salts, Sure's No. 1, 4; cottonseed oil, 4. The diet was supplemented with thiamine, 2 mg; riboflavin, 4 mg; pyri-

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[1] Slanetz, C. A., and Scharf, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 17.

[2] Patrick, H., and Morgan, C. L., *Science*, 1943, **98**, 434.

[3] Polskin, L. J., A thesis submitted to Rutgers University, New Brunswick, N.J., May, 1940.

[4] Jensen, J. L., Hickman, K. C. D., and Harris, P. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 294.

* This assay was made by Food Research Laboratories, Inc., Long Island City.

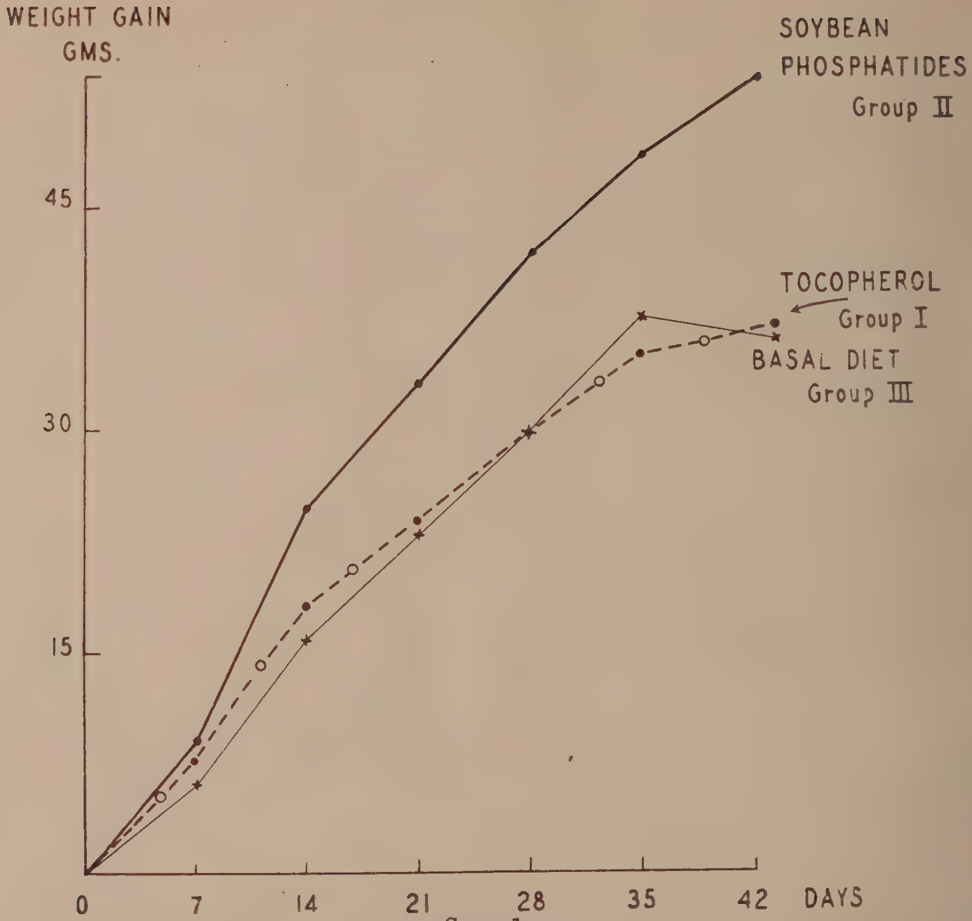

CHART 1.
Growth Curves of Rats.
Diet of Group I contained added alpha-tocopherol, of Group II soybean phosphatides of equivalent vitamin E content.

doxine, 4 mg; nicotinic acid, 1 mg; choline chloride, 500 mg; and 200 mg Viosterol per kg of diet. 100 μg of calcium pantothenate was given orally daily. After depletion of vitamin A, 3 groups of 10 rats each were fed the following diets for a period of 6 weeks in addition to 1.2 μg of carotene daily. Group I received the basal diet supplemented with 13 mg alpha-tocopherol per kg of diet. Group II was given the basal diet supplemented with 1% soy bean phosphatides;[†] Group III was fed the basal diet alone. The rats were weighed weekly during the test period. Chart I represents the average gain in weight for each group.

At the end of the test period the rats were continued on their respective diets and fed 6 μg of carotene daily. After 10 days some of each group were mated and examined for sperm. On the sixteenth day of pregnancy the rats were sacrificed and the results in regard to vitamin E activity were evaluated in part according to the formula outlined by Mason.[5] The rats receiving 1% soybean lecithin showed a normal gestation with no resorptions and uterine index above 3.00. The animals fed the basal diet with added tocopherol gave a negative response, with resorptions and a low uterine index.

[†] Soybean lecithin furnished by the American Lecithin Co., Elmhurst, N.Y.

[5] Mason, K. E., *J. Nutrition,* 1942, **23**, 59.

The remaining rats were kept an additional 10 days, then sacrificed and pooled samples of liver tissue and blood were obtained for vitamin A determinations. Blood from the soy bean lecithin-fed group of rats yielded vitamin A values more than 3 times as great as those of the other groups.

Discussion. The results shown in Chart I indicate that vitamin E is not the determining factor for the differences in growth. The animals fed tocopherol grew no better than those on the basal diet alone. Furthermore, the growth of both of these groups was inferior to that of rats given soybean lecithin. That the vitamin A metabolism is primarily involved is not only concluded from the experimental conditions but also by the appearance of the animals, showing a well defined vitamin A deficiency of the non-lecithin-fed controls regardless of the vitamin E intake. The rats fed lecithin showed no signs of vitamin A deficiency. The blood vitamin A findings are further confirmation of the observed differences in vitamin A metabolism.

The evaluation of vitamin E deficiency by Mason's formula shows that the unknown factor is essential for vitamin E metabolism also and that the rats, even after receiving adequate amounts of vitamin E, develop vitamin E deficiency if the unknown factor is absent. These results are in agreement with the findings of Patrick and Morgan.

Summary. Our earlier findings, that soy bean lecithin contains an unknown factor essential for the utilization of vitamin A, have been confirmed. It has been found that in the rat, as in the chick, vitamin E metabolism is also influenced by this factor.

14738

Simple, Standardized Culture Medium for Physiological Studies on *Entamoeba histolytica.**

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Since the successful cultivation by Boeck and Drbohlav[1] of *Entamoeba histolytica* in the presence of a bacterial flora, a valuable method has been available for the study *in vitro* of this important protozoan pathogen, especially in regard to diagnostic procedures. Other culture methods also have been developed.[2,3,4,etc.] These have in common the use of a solid nutrient slant and a fluid overlay containing an isotonic salt solution plus serum, or egg albumin, or other protein substances. There are several disadvantages in these procedures: (1) It is tedious to prepare culture tubes, especially in the case of egg-slants; (2) the fluid overlays must be sterilized by filtration owing to their coagulability upon heating; (3) the media become turbid owing to active growth of the bacterial flora, resulting in poorer visibility and limited life of the cultures; (4) the amebæ adhere to the slants and render quantitative counting difficult.

More recently Reardon and Rees[5] reported in a brief note that it was possible to omit the serum from the method of Boeck and

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* Aided by a research grant from the Graduate School of Northwestern University.

[1] Boeck, W., and Drbohlav, J., *Am. J. Hyg.*, 1925, **5**, 371.

[2] Dobell, C., and Laidlaw, P., *Parasitol.*, 1926, **18**, 283.

[3] Tanabe, N., and Chiba, E., *Acta med., Keijo*, 1928, **11**, 221.

[4] Cleveland, L., and Collier, J., *Am. J. Hyg.*, 1930, **12**, 606.

[5] Reardon, L., and Rees, C., *J. Parasitol.*, 1939, **25** (Suppl.), 1.

Drbohlav. Snyder and Meleney[6] have reported, without amplification, that they could replace previous methods of cultivation "by an entirely liquid medium prepared by infusing coagulated whole egg in 0.8% sodium chloride."

As part of a general program of one of us designed to study the cellular biology of *E. histolytica*, an attempt has been made to prepare a standardized culture medium which at the same time would avoid the above disadvantages and would be inexpensive and available in any experimental or diagnostic laboratory. Of the several leads followed, that first suggested by Snyder and Meleney proved best. The present paper reports the preparation of a standardized fluid culture medium from an infusion of coagulated egg yolk made up in a buffered salt solution.

It was necessary to consider a number of factors in infusing coagulated egg: the temperature of coagulation, the fineness of subdivision prior to infusing, the fractions of the egg employed, the "concentration" of egg substance employed, the medium in which the actual infusion occurs, etc. All these factors were investigated before the following basic procedure was established: To prepare 250 ml of culture medium, 2 eggs are hard-boiled for 15 minutes. Upon cooling, the egg whites are discarded and the yolks are crumbled in a beaker containing 125 ml of 0.8% sodium chloride solution. The mixture is boiled for 10 minutes, and after replacement of water of evaporation the infusion is filtered by suction and restored to 125 ml volume. The filtrate is autoclaved 20 minutes at 15 pounds pressure. Upon cooling, a slight precipitation of yolk settles, and is removed by simple filtration, after which 125 ml of M/15 phosphate buffer (pH 7.5) is added, making the total salt concentration M/30 phosphate solution in 0.4% sodium chloride. This final mixture is tubed in 5 ml amounts, autoclaved as before, and then is stored under refrigeration until use. Before introducing amebæ a bacterial loop of sterile rice starch is added to each tube. The sterile medium is almost completely transparent when prepared in this manner. It was found, on the other hand, that addition of buffer prior to the first autoclaving, or preparation of the infusion in distilled water, produced a densely turbid medium containing visible aggregates of egg protein.

Under these cultural conditions the growth of several strains of *E. histolytica* has proved uniformly good. Maximal growth is usually attained between the second and fourth days, while active, feeding amebæ persist on the average for more than nine days. The medium itself remains quite transparent throughout this period. The concentration of amebæ is less here, although not greatly so, than in the media of Boeck and Drbohlav or of Cleveland and Collier. This difference is due to the omission of additional nutrients (liver infusion, serum, etc.) and is more than compensated for in cyclical and cellular studies by the prolonged vigorous life of cultures.[†]

Modifications were sought in this basic medium to permit rapid acceleration of growth when required. The two most successful methods found were: (1) to double the concentration of egg yolks; and (2) to add Wilson liver concentrate powder 1-20[‡] in concentration of 0.5%. The concentration of egg yolk cannot be increased indefinitely owing to the tendency of its colloidal particles to settle out of solution. This particular liver concentrate gave the best results of the several tested. Use of either modification, or both together, increased the population count to the highest levels obtained with the media of other investigators.

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[6] Snyder, T., and Meleney, H., *J. Parasitol.*, 1943, **29**, 278.

[†] After submitting the present paper for publication, there has come to our attention the work of Rees, C., Bozicevich, J., Reardon, L., and Daft, F., *Am. J. Trop. Med.*, 1944, **24**, 189. In comparing media for their effects on the growth of *E. histolytica*, they reported that "... there were striking differences between the yields from whole egg, egg white, and egg yolk media, with the last two showing scanty growth." Since their whole egg and egg yolk media were coagula, the present report suggests that our egg yolk infusion may yield nutrients in a more available form than their egg yolk coagulum. This would explain the difference in relative effects on growth and would suggest further study of the nature of the biochemical factors involved.

[‡] Obtained through the courtesy of Dr. David Klein, Wilson Laboratories, Chicago, Ill.

The above modification, with both increased concentration of egg yolk and Wilson liver powder, is being tested in a Chicago hospital which cultures some 200 stools weekly. Comparative results indicate provisionally that with this culture medium: (1) there is a greater incidence of excystation of intestinal amebæ; (2) maximal growth of amebæ taken from fresh stools is *at least* as abundant as in other media; (3) the bacterial flora and *Blastocystis* do not grow as well in this medium.

Summary. The advantages claimed for the present culture medium for *E. histolytica* include the greater ease of preparation (and cleaning of used tubes), the prolonged life of cultures, its special suitability for studies on cellular biology, and its equal or superior adaptability for use in clinical diagnosis.

14739 P

The Anti-diamidine Activity of Sodium Nucleate.

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It has been shown recently[1,2] that aromatic diamidines which are active against protozoa *in vivo* and *in vitro* also possess antibacterial activity *in vitro* and are of value in wound therapy.

According to Fildes[3] the essential action of chemotherapeutic compounds lies in their interference with the functioning of vital cell metabolites. This interference may take the form of oxidation of the metabolites, molecular combination with it, or competition for the enzyme which acts on it.

Hawking and Smiles,[4] studying the distribution of stilbamidine in trypanosomes, found that this fluorescent substance tends to concentrate in the blepharoblast and in certain plasma granules. Adler and Tchernomoretz[5] noted that after treatment of certain babesia infections with stilbamidine, the first signs of degeneration occur in the nucleus of the parasites.

The present note describes an attempt to elucidate the mechanism of action of aromatic diamidines on bacteria *in vitro*.

The experiments were carried out with *B. coli* and *S. aureus* using two diamidines, stilbamidine (4:4-diamidino stilbene) and pentamidine (4:4-diamidino-diphenoxypentan). *B. coli* was grown on ammonium-phosphate, glucose, inorganic salts medium and *S. aureus* on the semi-synthetic medium of Knight.[6] The pH was 7.3-7.4, and incubation temperature 37°C. The standard inoculum was 0.1 ml of 1:10,000 dilution of a 24-h agar slant, in 5 ml of culture medium. Inhibition was defined by absence of growth after 48 hours. The sensitivity of *S. aureus* to both diamidines is 3-4 times as great as that of *B. coli*. Stilbamidine is less potent than pentamidine against either of the two test organisms.

The addition of peptone, meat extract, or marmite to the medium lessened or abolished the effect of the diamidines, the degree depending on the amount added. Serum (10%) did not show such an effect. Substances similar in structure to the diamidines: creatin,

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[1] Fuller, A. T., *Biochem. J.*, 1942, **36**, 548.

[2] Thrower, W. R., Valentine, F. C. O., McIndoe, A. H., Tilley, A. R., Morley, G. H., Bentley, J. P. M., Kohn, F., Hall, M. H., and Gross, C. D., *Lancet*, 1943, **1**, 133.

[3] Fildes, R., *Lancet*, 1940, **1**, 955.

[4] Hawking, F., and Smiles, J., *Ann. Trop. Med.*, 1941, **35**, 45.

[5] Adler, S., and Tchernomoretz, J., *Ann. Trop. Med.*, 1941, **34**, 199.

[6] Knight, B. C. J. G., *Biochem. J.*, 1937, **31**, 966.

creatinin, arginin, guanin, had no anti-diamidine activity. The following amino acids: glycine, *d,l*-methionine, *l*-tryptophan, *d,l*-phenylalanin, *d,l*-valin, *l*-leucin, *d,l*-serin, alanin, *d,l*-2-amino-n-valeric acid, *l*-oxyprolin, glutamic acid, *l*-prolin, histidin hydrochloride, lysin dihydrochloride, cystin hydrochloride, were likewise without effect.

The antibacterial activity of the diamidines was prevented when yeast sodium nucleate in suitable concentrations was added to the cultures. When sodium nucleate was added to a culture which had been maintained in a state of stasis for 24 hours by exposure to diamidine, growth developed within 24 hours.

The concentration of sodium nucleate which abolished stasis varied with the bacterial species and with the diamidine employed. Bacteriostasis induced by stilbamidine was abolished by a lower concentration of sodium nucleate than was that induced by pentamidine. A lower concentration of sodium nucleate abolished the diamidine effect on *B. coli* than that on *S. aureus*. Abolition of bacteriostasis of *B. coli* exposed to 1:25,000 stilbamidine was prevented by 0.05% sodium nucleate. Sodium nucleate of animal origin, prepared from embryo tissue, gave similar results. Separate constituents of nucleic acids (adenylic acid, adenin, guanin, uracil, xanthin, hypoxanthin) had no influence on the action of diamidines on *B. coli* and *S. aureus*.

The concentrations of nucleate required to neutralize the effect of the diamidines were rather high; however, they are of the same order as the amounts found in bacterial cells. Thus Thompson and Dubos[7] found 2-5% of nucleic acid, dry weight, in pneumococci, and Johnson and Coghill[8] 0.42% in *M. tuberculosis*.

In order to ascertain whether the anti-diamidine activity of sodium nucleate preparations was due to the presence of small amounts of accessory growth substances, the effect of the following vitamins of the B-complex in concentrations of 10-100 γ per ml was tested with negative results: thiamin, riboflavin, niacin, cholin, pimelic acid, calcium-pantothenate, biotin, pyridoxin, para-aminobenzoic acid, inositol.

Prof. Adler of the Department of Parasitology examined the effect of sodium nucleate on the stasis induced by stilbamidine on *L. donovani in vitro*. In the serum-semisolid medium containing 1:50,000 stilbamidine, growth in the presence of 1% sodium nucleate equalled that of the controls, whereas in the absence of sodium nucleate marked growth inhibition was noted.

Trichomonas vaginalis fails to grow in Locke-goat-serum medium containing stilbamidine in a concentration of 1:300,000. In the presence of 1% sodium nucleate growth occurs even in a stilbamidine concentration of 1:5,000.

In view of these results and of the fact that in protozoa diamidines tend to concentrate primarily within bodies rich in nucleic acids, it is suggested that diamidines as basic substances combine with nucleic acids and nucleoproteins, causing a blockage of these essential metabolites.

Summary. Experiments reported above show that sodium nucleate added to the substrate counteracts the inhibiting effect of diamidines on the growth of *B. coli*, *S. aureus*, *L. donovani*, and *Tr. vaginalis*.

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[7] Thompson, R. H. S., and Dubos, R. J., *J. Biol. Chem.*, 1938, **125**, 65.

[8] Johnson, T. B., and Coghill, R. D., *J. Am. Chem. Soc.*, 1925, **47**, 2838.

14740

Testicular Tumors in the Teleost (*Xiphophorus helleri*) Receiving Sesame Oil.

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Sesame oil is often used as a vehicle for fat-soluble endocrine preparations which gives the protracted action for free hormones. Pinkerton[1] suggested the use of this oil for roentgenologic purposes as it appears to have no reaction when introduced into the lungs. There are increasing evidences, however, that it may not be inert under some other conditions.[2-8] The possibility of induction of subcutaneous sarcomas when this oil is used as a hormone-solvent in injections has been indicated by Voegtlin.[9] Recently, Conrad *et al.*[10] reported a case of sesame oil tumors in the subcutaneous tissue of the arms of a woman who was treated with estrogen dissolved in the oil. Steiner *et al.*[11] found that overheated (350°C) sesame oil is carcinogenic, inducing 3 sarcomas in 9 mice that lived for more than one year.

In connection with another study of endocrine activities in the teleost, *Xiphophorus helleri,* a small control group of animals was injected with sesame oil only. Upon microscopical examination, a malignant testicular tumor of the interstitial cells had developed in two specimens and later a nodular hyperplasia of interstitial cells was noted in another. The present paper is concerned with studies of these interstitial cell tumors together with notes on some other effects of sesame oil injections, in an extended series of experiments.

Materials and Methods. In one series, 16 adult males were used, 8 of which served as untreated controls and the others were injected retroperitoneally with a weekly dosage of 0.02 cc sesame oil. Five animals received 4 injections each, and the other 3 animals were each subjected to 6 injections. They were killed 2 weeks after the last injection. In the second series 44 adult males were used, 12 of which were untreated controls. Thirty-two fish were each injected with 0.02 cc of sesame oil at intervals of 15 days. Six of them each received 27 or more injections. In both series, the fish were kept individually in separate aquaria. The visceral organs were removed as one mass at autopsy and fixed in Bouin's or Zenker's fluids. Serial sections were stained either with hematoxylin and eosin or Mallory's connective tissue stains.

Observations. Generally upon autopsies of the oil-injected fish a few droplets of oil were found in the peritoneal cavity. The omentum, which in pisces is usually undeveloped, was found to be filled abundantly with fat and small fat-nodules in prolonged treated fish. The lymph plexus also was well distended with fat. Histological examination revealed that large macrophages characterized by brownish yellow lipoid material were present in the peritoneum, spleen, mesonephros, and other visceral organs. Evidences of degenerative hepatitis and fatty liver were seen in some specimens. Normally the white, elongated testis is located in a position posterior to the intestine, just beneath the dorsal peritoneum. The posterior part of the testis generally shows more or less complete bilaterality, while the anterior part is small and single. Each lobe of the bifurcated testicle consists of a centrally placed sperm duct which usually contains many spermatophores, and tubules within which are seen the germ cells. Germ cells of the same spermatogenic stage are clustered together forming numerous cysts.[12] The interstitial cells as seen in sections are ordinarily very scanty and scattered, with blood vessels among the sperm ducts and tubules.[13]

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[1] Pinkerton, H., *Arch. Path.*, 1928, **5**, 380.

[2] Pollia, H. A., *Radiology*, 1937, **29**, 683.

[3] Spurr, C. L., and Kochakian, C. D., *Endocrinology*, 1939, **25**, 782.

[4] Tobin, C. E., *Endocrinology*, 1941, **28**, 419.

[5] Bruce, R. A., and Tobin, C. E., *Endocrinology*, 1940, **27**, 956.

[6] Clausen, F. W., *Endocrinology*, 1940, **27**, 989.

[7] Stein, K. T., and Allen, E., *Anat. Record*, 1942, **82**, 1.

[8] Crafts, R. C., *Endocrinology*, 1942, **31**, 142.

[9] Voegtlin, C., *J. Nat. Cancer Inst.*, 1942, **2**, 309.

[10] Conrad, A. H., Conrad, A. H., Jr., and Weiss, R. S., *J. Am. Med. Assn.*, 1943, **121**, 237.

[11] Steiner, P. E., Steele, R., and Koch, F. C., *Cancer Research*, 1943, **3**, 100.

The first tumor was observed to occur in a fish which had received only 6 injections. This testis was slightly enlarged, the bifurcated halves now fuse so that in transverse sections the gonad appeared single. A large portion of the sperm tubules were now replaced by the tumor cells with an indefinite limiting margin of fibroblasts. Thus the tumor cells merged with the surrounding interstitial cell hyperplasia and the remaining spermatocysts. The partially atrophied sperm ducts either contained atypical released spermatozoa or were entirely empty. The tumor cells which were identified as interstitial cells were polygonal in shape with a single nearly rounded vesicular nucleus. Many of these cells, however, were vacuolated and only rarely showed mitotic figures. Blood vessels were rare and small foci of yellow-colored macrophages were observed among the tumor cells, and were infiltrated into the mesorchium but no other metastases were found.

In the second series of experiments, a malignant testis tumor occurred in a fish which had received 23 injections. The testis was a single, greatly enlarged mass and the tumor was similar to that just described. Many small empty cysts which marked the atrophic sperm-tubules were often surrounded by fibroblasts. As layers of fibroblasts inclosed the lumen of sperm ducts which contained mainly the debris of necrotic epithelial cells, the whole structure appeared like a lymph nodule. Blood vessels and macrophages were more abundant than in the previous case. Mitosis of tumor cells was rare and many cells showed foamy cytoplasm or necrosis. No metastases except in the mesorchium were seen. Cysts containing necrotic cells, macrophages, and lymphocytes were found within the lobules of the dispersed pancreas on the fatty mesentery.

In the testicle of a fish which had received 23 injections, a considerable number (7) small nodules of interstitial cell hyperplasia were discovered. These nodules were located either on the periphery or near the sperm ducts. The largest nodular mass was 0.2 mm in diameter. Each nodule consisted of many foamy tumor cells and was well demarcated from the rest of the testicular tissue. Cysts of necrotic cells were again seen in the pancreatic lobules of this fish and a mild degree of fatty liver was also observed.

Discussion. Jemerin[14] reported a case of interstitial-cell tumor of the testicle in man and critically reviewed the previous records of the similar hyperplasia and neoplasia in man as well as in horses and dogs. Recently, several additional cases of interstitial-cell tumors of the human testicle were noted by Bonser and Hawksley[15] and Warren and Olshausen.[16] The latter workers classified the growths into 3 groups: hyperplasia, local nodular tumor, and malignant tumor. Testicular tumors appear to occur more frequently in animals other than man.[17] Innes[18] examined testicle-tumors from 1 calf, 2 horses, and 49 dogs, of which 3 cases were identified as the interstitial-cell tumors. Besides, he observed 12 cases of nodular hyperplasia of interstitial cells in old dogs. It is well known that both benign and malignant tumors of interstitial cells may be induced in certain strains of mice when treated with estrogens.[19] Thus far no previous descriptions of the testicular tumor in teleost have been noted. Since the tumors were not found in normal untreated fish in our laboratory the question of hereditary predisposition of the neoplasm cannot be answered at the present.

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[12] See pl. 5, Baldwin, F. M., and Goldin, H. S., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 813.

[13] Baldwin, F. M., and Li, M. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 601.

[14] Jemerin, E. E., *Arch. Surg.*, 1937, **35**, 967.

[15] Bonser, G. M., and Hawksley, L. M., *J. Path. and Bact.*, 1943, **55**, 295.

[16] Warren, S., and Olshausen, K. W., *Am. J. Path.*, 1943, **19**, 307.

[17] Ewing, J., *Neoplastic Diseases*, 4th ed., p. 854, 1940.

[18] Innes, J. R. M., *J. Path. and Bact.*, 1942, **54**, 485.

[19] For example: Bonser, G. M., *J. Path. and Bact.*, 1944, **56**, 15; Gardner, W. U., *Cancer Research*, 1943, **3**, 92; Hooker, C. W., and Pfeiffer, C. A., *Cancer Research*, 1942, **2**, 759; Shimkin, M. B., and Andervont, J. B., *J. Nat. Cancer Inst.*, 1942, **2**, 611.

The tumor-producing action of corn oil on tissue has been investigated by Burrows and Johnston.[20] Literature reveals that sesame oil may act as a stimulator or inhibitor to certain physiologic activities of tissues or organs. Caspari and Ottensooser[21] claimed that peroral administration of this oil may even impede the growth of transplanted tumors. The chemical nature of sesame oil has been discussed by Bruce and Tobin.[5] It seems that the actions of this oil might be due to some inclusions in the oil. Further studies now in progress using extracted fractions of this oil should help to settle the problem.

Summary. Interstitial cell tumors of the testicle occurred in 3 of 40 adult male teleost (*Xiphophorus helleri*) that had received retroperitoneal injections of sesame oil. Two of the tumors were malignant and the other was local nodular hyperplasia. Damage to other organs and some other effects of the oil treatment were observed. No neoplastic changes were found in the untreated adult male fishes.

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[20] Burrows, M. T., and Johnston, C. G., *Arch. Int. Med.*, 1925, **36**, 293.

[21] Caspari, W., and Ottensooser, F., *Z. f. Krebsforsch.*, 1930, **32**, 74; 1932, **38**, 351 (Stern, K., and Willheim, R., *The Biochemistry of Malignant Tumors*, p. 406, p. 450, Reference Press, N.Y., 1943.)

14741

A Difference in Cardiodecelerator Action Between Digitoxin and Digitoxigenin.*

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The cardiodecelerator action of the cardiac glycosides is not due to a single mechanism. A direct effect upon the mammalian heart, demonstrable in the isolated denervated organ, as well as effects mediated by the nervous system, play a role. One of the established sites of action involving the vagal mechanism is the carotid sinus, as was shown by Heymans and Heymans.[1] A central vagal stimulation was assumed by Cushny.[2] However, direct experimental evidence for it rests only on experiments by Greene and Peeler[3] on the innervated turtle heart in which the head was included in a perfusion system separate from that of the heart; while in dogs Heymans and Heymans were unable to observe a central vagus stimulating action of strophanthin when this was applied to the central nervous system in cross-circulation experiments.

Recently Walther[4] has drawn attention to a pharmacological difference between digitoxin and its aglucone, digitoxigenin. In electrocardiographic studies in intact cats, he noticed an immediate decrease in heart rate after the intravenous administration of digitoxigenin. No such change occurred after digitoxin.

Employing the innervated isolated heart of the dog in a cross-circulation method in

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[1] Heymans, J. F., and Heymans, C., *J. Pharm. and Exp. Therap.*, 1926, **29**, 203; *Arch. internat. de Pharmacodyn. et de Thérap.*, 1926, **32**, 9.

[2] Cushny, A. R., *J. Exp. Med.*, 1897, **2**, 233. For complete literature, see Lendle, L., in Heffter's *Handbuch der Pharmakologie. Erganzungswerk*, 1935, **1**, 161.

[3] Greene, C. W., and Peeler, J. O., *J. Pharm. and Exp. Therap.*, 1915, **7**, 591.

[4] Walther, R., *Arch. f. exp. Path. u. Pharmakol.*, 1940, **195**, 709; 1943, **201**, 611.

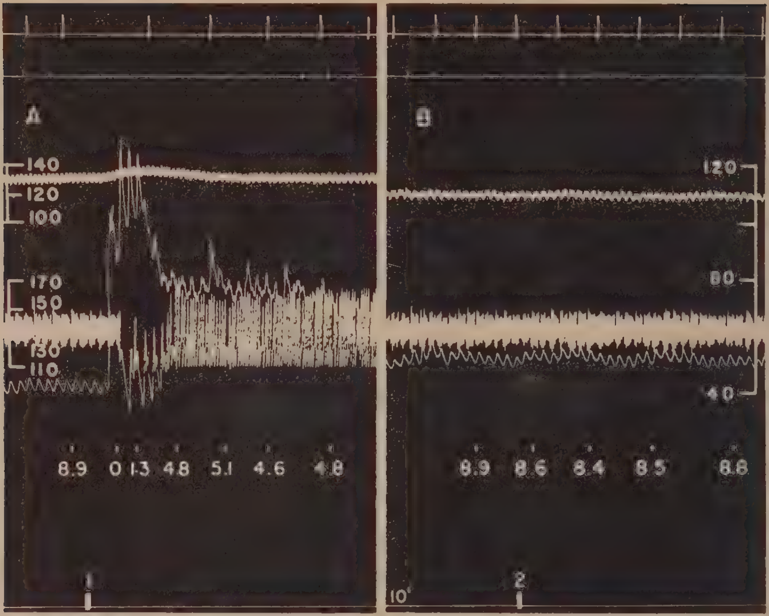

FIG. 1.

Experiment June 8, 1944. The effect of digitoxigenin and digitoxin upon heart rate. Innervated heart-lung preparation; cross-circulation. Head perfused from donor dog. Donor dog, male, 21.2 kg. Heart-lung dog, male, 12.0 kg. Chloralose anesthesia, 0.09 g per kilo. The blood of both animals was heparinized with 3 mg heparin per kilo. The donor received in addition a continuous infusion of heparin into the right femoral vein at a rate of 1 mg per minute. The rectal temperature of the donor dog was 37.3°C. The temperature of the blood in the heart-lung preparation was 38.2°C. The tracings from top to bottom indicate: output of heart-lung preparation in 100 cc; time in 10-second intervals; blood pressure of head region, scale at left in mm Hg; arterial pressure of heart-lung preparation, scale at left in mm Hg; right auricular pressure of heart-lung preparation, scale at right in mm water; signal line. The horizontal row of figures indicates heart rate in 5-second intervals taken from ECG. The zero means that the heart was arrested for 4.5 seconds. Between A and B there was an interval of 10 minutes.

At 1, injection of 0.2 mg of digitoxigenin in 0.1 cc. Blood flow to head 430 cc per minute. Drug concentration approximately 1:75,000. At 2, injection of 0.3 mg of digitoxin in 0.15 cc. Blood flow to head 430 cc per minute. Drug concentration in blood approximately 1:50,000. The injections were made into the arterial supply of the recipient head.

which the circulation of the head was kept separate from the heart-lung circuit, we made observations similar to those of Walther and were able to investigate the site of the cardiodecelerator action of digitoxigenin. Five experiments were performed which uniformly revealed the effect illustrated in Fig. 1.

The details of the type of method used were described by Krayer and Verney.[5] In two of the present experiments the perfusion of the head of the recipient dog was made from one carotid artery of the intact donor dog. The blood returned to the donor dog through one external jugular vein. The cardiac glycosides and genins were dissolved in a mixture of equal volumes of absolute ethyl alcohol and 0.9% sodium chloride solution. The injections were made into the arterial supply of the recipient head. The total amount of the solution injected was between 0.05 and 0.2 cc. The temperature of the solution was approximately 38°C at the time of injection, and the pH of the solution was approximately 7.0.

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[5] Krayer, O., and Verney, E. B., *Arch. f. exp. Path. u. Pharmakol.*, 1935, **180**, 75. See also Krayer, O., Wood, E. H., and Montes, G., *J. Pharm. and Exp. Therap.*, 1943, **79**, 215.

Results. Digitoxigenin injected into the head circulation of the recipient dog in doses of 0.05 to 0.2 mg exerted an influence upon the heart which was transmitted by way of the vagus nerves and resulted in a transient slowing of the heart rate. This effect was not dependent upon a blood pressure increase in the head, as could be shown by including in the head circulation a compensating device which prevented pressure changes. Furthermore, if in an uncompensated system a blood pressure increase of about 10 mm Hg did occur (as in the experiment of Fig. 1) the accompanying heart rate decrease was much more pronounced than that caused by a mechanical increase of the blood pressure in the head of 20 to 40 mm Hg. The type of anesthetic agent used proved to be of importance for an investigation of the quantitative relation between dose and effect. When pentobarbital sodium was employed, the action was much less pronounced than with the animals kept under chloralose.

This effect of digitoxigenin cannot be explained by a sensitization of muscular elements of the heart to vagal impulses in the sense of Straub,[6] as the method used separates the head circulation from the heart-lung circuit. Denervation of the carotid sinus areas, which were included in the head circuit, did not abolish the effect. Therefore the action cannot be identical with that of the cardiac glycosides upon the carotid sinus reported by Heymans and Heymans[1] and by Hering.[7]

The site of this action must be in the central nervous system, and it is well to remember in this connection that digitoxigenin is a convulsant agent.[8] It is possible that this central vagal action is a property of the aglucones of the cardiac glycosides. In a single experiment with gitoxigenin, equimolar amounts showed an effect similar to that of digitoxigenin, but less in intensity.

The central stimulating effect upon the vagus of the mammal caused by digitoxigenin and gitoxigenin is not a property of the corresponding cardiac glycosides. Digitoxin, even in larger than equimolar doses, did not cause any significant change in heart rate, irrespective of whether the injection of the glycoside preceded or followed the injection of the aglucone. Gitoxin, which was compared with gitoxigenin, also was without effect in equimolar doses. That strophanthin had no central stimulating effect upon the vagus was already shown in earlier experiments.[1,9]

In interpreting the results of experiments reported in the older literature one has to take cognizance of the fact that impure preparations were often used—as, for instance, in the experiments of Greene and Peeler with soluble digitalis Mallinckrodt—and it is not improbable that such impure preparations were mixtures of glycosides and genins.

Summary. There is a qualitative difference between the circulatory action of digitoxin and that of its aglucone, digitoxigenin. Digitoxigenin causes an immediate and transient cardiodeceleration due to a central stimulation of the vagus. Digitoxin is without effect if administered in equimolar doses under the same experimental conditions. This pharmacological difference between digitoxin and its genin was demonstrated and analyzed with the use of the innervated heart-lung preparation of the dog in which the drugs could be administered into the blood supply to the central nervous system without reaching the heart-lung circuit.

The digitoxigenin and gitoxigenin used in our experiments were prepared by R. P. Linstead and D. Todd, from the Department of Chemistry, Harvard University. The digitoxin and gitoxin were generously supplied by Burroughs Wellcome, Ltd., London. The pharmacological experiments were carried out with the help of Dr. Rafael Mendez and Dr. Gordon K. Moe.

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[6] Straub, W., Heffter's *Handbuch der Pharmakologie,* 1924, **2**, part 2, 1419.

[7] Hering, H. E., *Arch. f. d. ges. Physiol.,* 1924, **206**, 721; *Z. Kreislaufforschung,* 1930, **22**, 287.

[8] Trendelenburg, P., Heffter's *Handbuch der Pharmakologie,* 1920, **2**, part 1, 427.

[9] Heymans, C., Bouckaert, I. I., and Regniers, P., *Arch. internat. de Pharmacodyn. et de Thérap.,* 1932, **44**, 31.

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